ß-LACTAMASE-MEDIATED RESISTANCE IN NOSOCOMIAL GRAM NEGATIVE AEROBIC BACILLI

by

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Success is to be measured, not so much by the position that one has reached in life, as by the obstacles which one has overcome while trying to succeed.

Booker T. Washington

DEDICATION

To the treasured memory of my father, Robert Paton, who passed away during the final few months of this thesis. He would have been so proud.

Abstract

A survey of antibiotic resistance on Gram negative aerobic bacilli which had been isolated from blood cultures during the period 1980-1991 from Edinburgh Royal Infirmary was performed. All viable cultures were investigated for susceptibility to an extensive range of antibiotics including the newer carbapenems, imipenem and meropenem.

Gram negative, oxidase negative, aerobic bacilli (245) that were found to be resistant to cefuroxime (>4.0 mg/l) were investigated further. As expected, the predominant species present were: *Enterobacter*, *Serratia* and *Acinetobacter*. Isoelectric focusing (IEF) and conjugation studies were performed. It was clear that most of these strains possessed chromosomal cephalosporinases. None of the strains that transferred ampicillin resistance to *Escherichia coli* J62-2 co-transferred resistance to any of the later generation cephalosporins or to imipenem. It was apparent that inducible chromosomal ß-lactamases were the main cause of cefuroxime resistance in these strains.

Amongst the cefuroxime resistant strains an isolate of *Acinetobacter baumannii* was found to be resistant to all ß-lactams, including imipenem and meropenem. Isoelectric focusing revealed the presence of a chromosomal cephalosporinase and a novel ß-lactamase of pI 6.65. Despite the fact that the original clinical isolate could be cured of its resistance to carbapenems and penicillins by growing in the presence of ethidium bromide with the concurrent loss of the enzyme of pI 6.65, no resistance plasmid was transferred. Plasmids were isolated but the physical loss of a plasmid in the cured strain could not be demonstrated. The enzyme hydrolysed penicillin, ampicillin and cephaloridine slowly during enzyme assay but inactivation of carbapenems could only be demonstrated by microbiological means. The enzyme was thus designated ARI-1 (Acinetobacter resistant to imipenem). The molecular mass of the ARI-1 enzyme was 23 kDa. It was inhibited by BRL 42715 indicating that it was a serine active site ß-lactamase. It was not inhibited by EDTA.

The susceptibility profile and ß-lactamase complement of 28 strains of *Xanthomonas maltophilia* isolated from blood cultures were examined. Most strains were resistant to a wide range of antimicrobials including carbapenems, although the level of resistance to ß-lactams was found to be dependant on the sensitivity testing media employed.

The ß-lactamase complement of these strains was studied further. No discernible ß-lactamase activity was present without prior induction of enzyme. Isoelectric focusing revealed the heterogeneous nature of ß-lactamase production amongst these isolates with several ß-lactamases identified by virtue of their isoelectric point. Contrary to a previous report most strains appeared to code for two or more ß-lactamases.

These results indicate the diversity of ß-lactamases amongst this species.

Seven of the strains displayed identical ß-lactamase profiles, designated XMCE type-1 (*Xanthomonas maltophilia* chromosomal enzyme), these were studied further by standard methodology with the aim of characterising them fully. On IEF, all seven isolates demonstrated several bands commensurate with major ß-lactamase activity of between pI 5.2-6.8 when stained with nitrocephin. Strain 5B105 was chosen as representative. It was found that these strains encoded for the production of at least four ß-lactamases. One was an inducible penicillinase of pI 6.8 with potent carbapenemase activity. The approximate molecular mass (M_r) was 96kDa by Sephadex gel filtration and 26kDa by SDS-PAGE, suggesting that this enzyme was a tetramer consisting of four subunits of equal size. The enzyme was completely inhibited by EDTA, but enzymatic activity was recovered by the addition of zinc sulphate. It was not inhibited by BRL 42715. The other enzymes exhibited isoelectric points of pI 5.3, 5.55, and 6.2. The M_r of all bands of ß-lactamase activity was 48kDa by gel permeation and a single protein of M_r 24kDa was visualised by SDS-PAGE, indicating dimeric enzymes in their native state. They were inhibited by BRL 42715 and partially inhibited by clavulanate suggesting they were serine active site ß-lactamases. Kinetic studies demonstrated that together they hydrolysed penicillins and cephalosporins but not carbapenems or the monobactam, aztreonam.

The similarities of these serine active site ß-lactamases with the ubiquitous TEM plasmid-mediated enzymes did not go unnoticed. The hypothesis that the serine ß-lactamase present in the latter strains of *Xanthomonas maltophilia* may have an ancestral relationship was investigated. Chromosomal DNA was prepared from *X. maltophilia* 5B105 and Polymerase Chain Reaction (PCR) was performed with universal DNA primers for the TEM gene, to determine whether the structural gene for TEM was present and could be amplified. The hypothesis was proven to be unfounded.

Finally future directions for further research are examined.

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DECLARATION

The experiments and composition of this thesis are the work of the author unless otherwise stated.

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PUBLICATIONS

Full Papers

Paton, R., Miles, R.S., Hood, J., & Amyes, S.G.B. (1993). ARI 1: β-lactamase-mediated imipenem resistance in *Acinetobacter baumannii*. *International Journal of Antimicrobial Agents* **2**: 81-88.

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Thomson, C.J., Paton, R., Hood, J., Miles, R.S., & Amyes, S.G.B.. (1992). Antibiotic resistance in urinary bacteria isolated in central Scotland. *International Journal of Antimicrobial Agents* **1**: 223-228.

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Abstracts that formed the basis of poster presentations

Paton, R., Hood, J., Miles, R.S., & Amyes, S.G.B. (1992). Antibiotic resistance in Gram negative aerobic bacilli isolated from blood cultures during 1980-91. *International Congress on the Management of Infection*, Amsterdam, Holland, Abstract no P1.41.

Paton, R., Miles, R.S., & Amyes, S.G.B. (1993). Four novel discrete β-lactamases responsible for imipenem resistance in *Xanthomonas maltophilia*. *The 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy*, New Orleans, USA (17-20 October) Abstract no 1521, p397.

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Abbreviations

Ap	Ampicillin
bla	ß-lactamase gene
bp	base pair
BP	breakpoint
cfu	colony forming units
Clav	clavulanate
DM	Davis and Mingioli media
DNA	deoxyribonucleic acid
DST	diagnostic sensitivity test medium
EDTA	ethylenediaminetetraacetate
ESBL	extended-spectrum ß-lactamase
FPLC®	Fast Protein Liquid Chromatography®
g	acceleration due to gravity
IEF	Isoelectric focusing
ID_{50}	concentration required to inhibit 50% of enzyme activity
his	histidine auxotrophy
IST	isosensitest medium
ITU	intensive therapy unit
kb	kilobase
kDa	kilodaltons
K_m	Michaelis constant
λ	wavelength of light
L	litres
M	Molar
mA	milliamperes
MH	Mueller Hinton medium
MIC	minimum inhibitory concentration
mg	milligram
mM	millimolar
µg	microgram
µM	micromolar
M_r	molecular mass
OD	optical density
OMP	outer membrane protein
PAGE	polyacrylamide gel electrophoresis

PBP	penicillin binding protein
PCR	Polymerase Chain Reaction
pI	Isoelectric point
pro	proline auxotrophy
R	resistant
RIE	Royal Infirmary of Edinburgh
Rif	rifampicin
S	sensitive
SDM	stably derepressed mutants
SDS	sodium dodecyl sulphate
Sm	streptomycin
Te	tetracycline
TEMED	tetramethylethylenediamine
Tp	trimethoprim
Tris	tris(hydroxymethyl)methylamide
trp	tryptophan auxotrophy
UV	ultra-violet light
V_{max}	maximum rate of hydrolysis
V	volts
W	watts

CHAPTER 1 INTRODUCTION

1.0. The Pre-antibiotic era: - Historical perspective

1.1. Introduction

What exactly is an antibiotic? Chambers Twentieth Century dictionary gives the following definition:

"Inhibiting the growth of another organism, used especially of a substance produced by micro-organisms which, in dilute solution, has the capacity to inhibit the growth of, or to destroy, micro-organisms causing infectious diseases".

Selman Waksman is generally regarded as the instigator of the word antibiotic. In 1942, he was asked to suggest an appropriate term describing the plethora of antimicrobial compounds that were appearing with increasing frequency in the medical-scientific literature. It appeared in *Index Medicus* in 1943. Waksman's original definition was very similar to the above:

"A compound produced by one micro-organism which is capable of killing or inhibiting another".

These definitions are unsatisfactory. They do not include synthetic compounds such as trimethoprim, sulphonamides and man-made modifications of microbial products, e.g. semi-synthetic penicillins and cephalosporins. Lietman, in 1986 [1], thought it more appropriate to redefine an antibiotic as:

"Any chemical substance produced by various micro-organisms or by humans and having the capacity, in dilute solutions, to inhibit the growth of or to destroy bacteria and other microorganisms".

Paul Ehrlich is credited with the term "magic bullet", used to describe a compound with a seemingly magical effect on bacterial infections, and targeted towards a specific pathogen.

Salvarsan, the first of the "magic bullets", was discovered by Ehrlich and Hata in the summer of 1907 [2], and caused great excitement, because of the potential ability to cure syphilis, yaws and other *Spirillum* infections. Because of severe toxicity associated with the drug, salvarsan was far from ideal, and unfortunately, was only

effective against this single group of pathogens. The search was launched to find more versatile (and safer) compounds. It was almost 25 years before the next breakthrough, and physicians sought alternative approaches to the treatment of bacterial infections.

Ample evidence exists that mould therapy was used from antiquity to relatively modern times to treat bacterial infections. The remains of a Sudanese tribe from around 350 AD, were found to contain traces of tetracycline (an antibiotic not discovered until the mid-1940s). The most likely source was that tetracycline-producing *Streptomyces* contaminating the food grains from which they made their bread [3].

Plants were also a rich source of antimicrobial substances. Cinchona bark was used in Peru to treat malaria, and ipecacuanha was found to be of use against amoebic dysentery [3].

In the 1930s, maggot therapy was used in an attempt to cure osteomyelitis [4]. It was originally assumed that maggots removed wound debris, but William Baer a US surgeon believed that maggots produced antibacterial, as well as wound healing secretions [4]. In 1935, S. W. Simmons demonstrated that maggot washings contained substances that killed organisms [5]. The nature of these substances has yet to be elucidated.

In 1976, a 67 year old man suffering from mastoiditis, was treated with maggots introduced into the mastoid cavity [6]. The infection, caused by *Pseudomonas* spp, *Proteus mirabilis* and enterococci responded well. All conventional therapy had failed. The authors concluded:

"The intractable nature of this man's infection makes it clear that, without maggot therapy, healing would not have occurred. The authors recognise that this unusual form of therapy is not part of the standard armamentarium against infections of the temporal bone. We see no need to categorically discard successful techniques of the past, however, because one never knows when they might be useful in the future. We feel justified in using this otherwise antiquated technique in a situation in which modern technology was inadequate".

1.2. The dawn of antimicrobial chemotherapy

Penicillin, isolated originally from the fungus *Penicillium notatum*, was discovered by Fleming in 1928 [7]. It was the first and arguably remains the most important of all the antibiotics. Its discovery revolutionised the treatment of pneumonia, scarlet fever, bacterial meningitis and septicaemia. The therapeutic power of penicillin was to remain unrecognised until 1941 when it was "rediscovered" by Florey, Chain and Heatley.

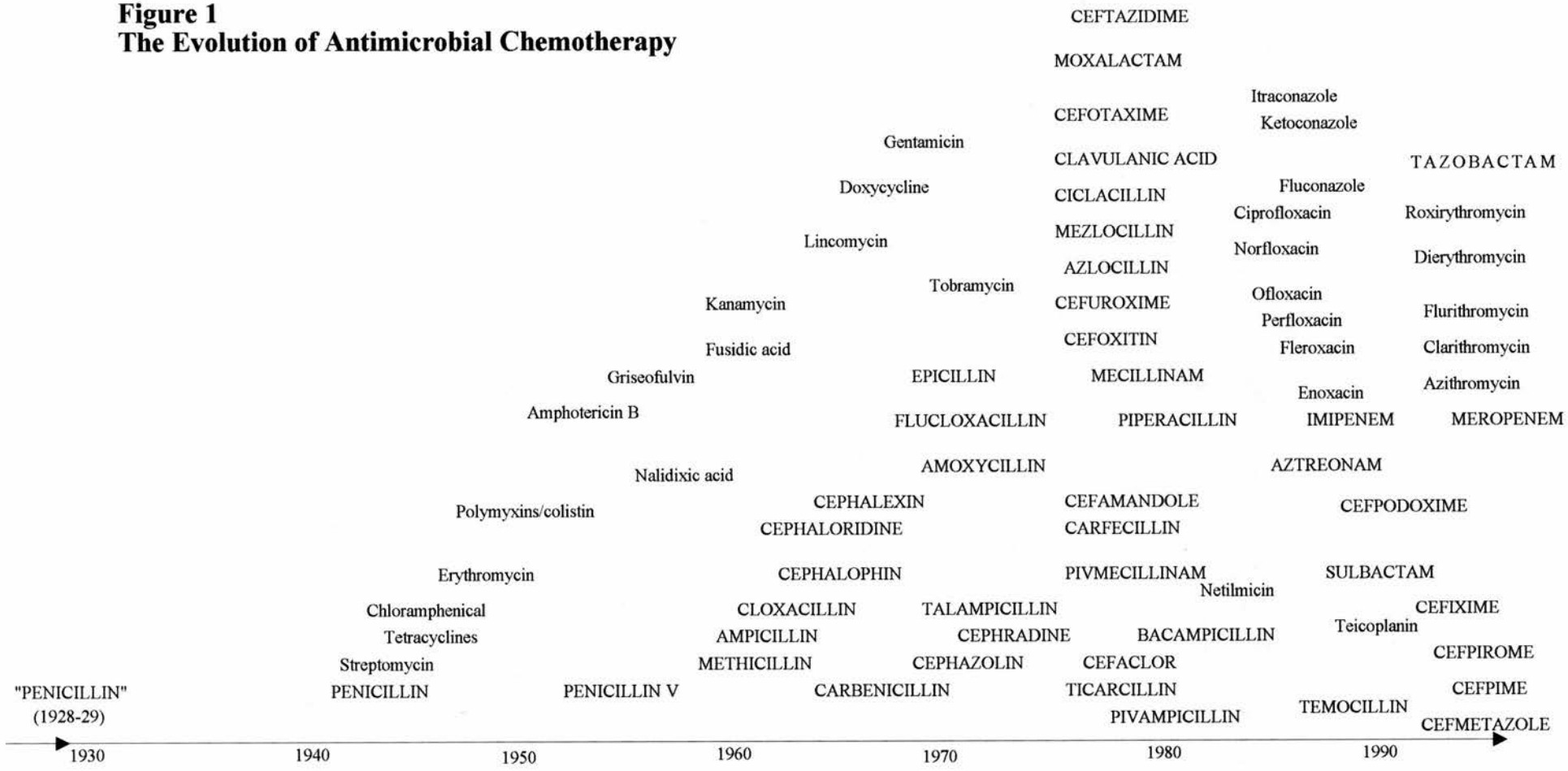
Although penicillin is generally regarded as the first antibiotic, it was in fact, preceded by a compound isolated from, *Bacillus brevis* [8]. This antibiotic, discovered by Rene Dubois, was named tyrothricin. It was later split into two pure antibacterial substances, which were named gramicidin and tyrocidin [9]. These compounds were too toxic for injection into the blood stream, but were found to be useful in the control of local infections.

Garrod [10] argued that the modern era of antimicrobial therapy was initiated in 1932 when Domagk first reported the protective effect of prontosil (the forerunner of the sulphonamides) against murine streptococcal infection. It was to be another three years before prontosil was released for general use [11].

Soon after the discovery of penicillin, research into other antibiotic producing micro-organisms was initiated all over the world. The evolution of antimicrobial chemotherapy is summarised in a table by Selwyn [12], updated by Hood [13] (see figure 1).

It is noteworthy, that of the hundreds of compounds discovered capable of inhibiting bacterial pathogens, very few were clinically useful. Fleming's discovery, although unrealised at the time, would turn out to be very special indeed!

Figure 1
The Evolution of Antimicrobial Chemotherapy



1.3. The discovery of penicillin

When Alexander Fleming left his laboratory at the end of July 1928, he was unaware he was due to make an historic discovery. In September, Fleming returned to his laboratory. One of the dishes that he had inoculated previously, caught his eye.

Fleming observed that a contaminating mould was inhibiting the growth of colonies of *Staphylococcus aureus* he had previously inoculated onto the plate. The colonies took on a "ghost" like appearance, indicating that the mould was causing the bacteria to lyse. The mould was originally misidentified as a strain of *Penicillium rubrum* by the mycologist, C. J. La Touche, although he was later to apologise for his error. The mould was subsequently correctly identified as *Penicillium notatum*.

Fleming realised that the mould was producing an antibacterial substance, which he named, penicillin. He manufactured crude extracts of penicillin by fermenting *Penicillium notatum*, and performed a number of early experiments on his "mould juice", but lacked the knowledge or expertise to purify, concentrate or store penicillin. In 1932, Fleming performed what was originally thought to be the first successful therapeutic use of penicillin, on his laboratory assistant, K. B. Rogers, who had been suffering from a pneumococcal eye infection.

In 1930, C. G. Paine, a hospital bacteriologist conducted some experiments on eye infections due to staphylococci, gonococci and pneumococci. This work was never submitted for publication. He was later to write (quoted by Wilson) [14]:

"The variability of the strain of *Penicillium* and my transfer to a different line of work led me to neglect further investigation of the possibilities of penicillin, an omission which, as you may well imagine, I have often regretted since."

In 1986, the English microbiologist, Milton Wainwright and Dr H. T. Swan, an ophthalmologist who had worked with Paine, discovered the original medical records [15]. There were two case notes, which confirmed that Paine used penicillin as early as 1930. The first of the case notes refers to a 3-week old baby called Peter who was suffering from bilateral gonococcal ophthalmia. The case notes read as follows:

Name	Peter xxxxxx
Disease	R & L ophthalmia neonatorum
On admission	Loose membrane L inner lid & top R
	Cornea hazy R & L
Culture	Gonococci +
25.XI. 30	Started Penicillin
2. XII. 30	Both eyes clean
11.XII.30	Home

Paine was to receive recognition, albeit 56 years later, of his early work, and he was awarded an honorary degree of Doctor of Medicine by Sheffield University. No more work of any note was performed on penicillin until 1940, when Howard Florey, Ernest Chain and the other members of the "Oxford" team performed the first therapeutic trials with partially purified penicillin. This led to their first publication on the properties of penicillin in 1940 [16].

By the end of 1940, the Oxford group had produced enough penicillin to commence clinical trials on a few patients. One of the first recipients was a 43 year old policeman, Albert Alexander, who was infected with staphylococci and streptococci. He was initially administered 200mg of partially purified penicillin, followed by injections of half this dose every two hours. His condition improved until the supplies of penicillin ran out. The setback of his subsequent death did not deter the British pharmaceutical companies from becoming involved.

Large scale production of penicillin was attempted, although it soon became obvious that the best hope for large scale production lay with the Americans. Scientists in Peoria, Illinois, were to make two vital contributions. First the discovery that deep fermentation techniques produced far greater yields than the shallow fermentation used by British companies. Second the discovery of a new strain of Fleming's mould, *Penicillium chrysogenum*. This strain was "mutated" by the Americans to produce vast amounts of penicillin.

The first therapeutic use of penicillin in a war zone by Major R. J. V. Pulvertaft took place in 1942 [17]. This was the final stimulus required for penicillin production. By 1944, supplies of penicillin to Allied troops were described as "unlimited".

1.4. The impact of 6-aminopenicillanic acid (6-APA)

Work carried out on *P. chrysogenum* in the 1940s demonstrated that other forms of naturally occurring penicillins were produced by fermenting *P. chrysogenum*. None of these was superior to Penicillin G [18], although Penicillin V was more acid stable and better absorbed when given orally.

In the late 1950s, Beecham Research Laboratories developed the production of large quantities of 6-APA [19] (figure 2), the core structure of penicillin. This allowed the addition of novel side chains, and development of new penicillins.

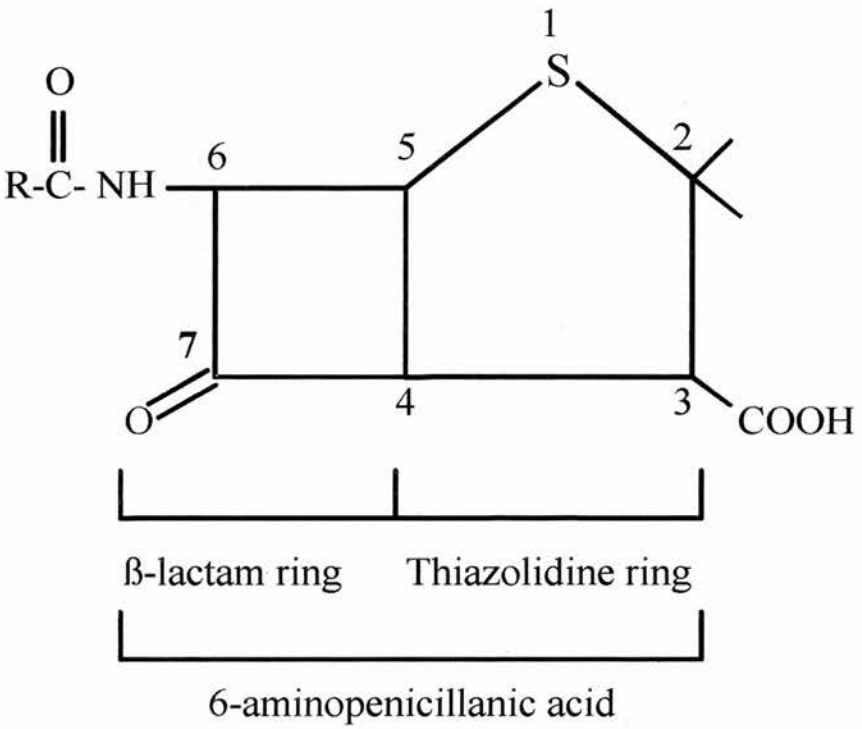
Benzylpenicillin still remains the first choice for a variety of bacterial infections, particularly those caused by the pyogenic cocci [20], but resistance *has* eroded the usefulness of penicillin. Ninety five per cent of hospital isolates of *S. aureus* and 85 per-cent of community isolates of *S. aureus* produced penicillinases that conferred resistance to penicillin [21].

1.5. The semi-synthetic penicillins

Modification of the ß-lactam nucleus to yield ß-lactamase stable antibiotics takes place on the acyl side chain attached to the C_6 carbon (figure 2).

To combat penicillin resistant staphylococci the first of the semisynthetic penicillinase-stable penicillins; methicillin and its derivatives, e.g. flucloxacillin were developed. The addition of an acyl side chain prevented disruption of the ß-lactam ring. These drugs remain first choice therapy in most staphylococcal infections, although the re-emergence of methicillin resistant *S. aureus* (MRSA) in the early 1980s has made in-roads into their usefulness. The need for penicillins with greater activity against Gram negative bacilli prompted the development of the aminopenicillins, carboxypenicillins and ureidopenicillins. Initially ampicillin was developed, followed by the extended-spectrum penicillins, e.g. carbenicillin, ticarcillin, azlocillin and piperacillin.

Figure 2



Chemical structure of penicillins and 6-aminopenicillanic acid

The resulting large family can be conveniently divided into six groups [22] (figure 3).

The penicillins have one drawback; they possess only one site on the molecule available for substitution (C_6), they have not, therefore, enjoyed the increased variety and continued success of the cephalosporin antibiotics.

Figure 3. From Lambert and O'Grady [22]

Naturally ocurring penicillins and penicillins derived from N-acyl derivatives of 6-APA

Group1 Benzyl penicillin and its long acting parenteral forms	**Group 2 Orally absorbed penicillins resembling benzylpenicillin**	**Group 3 Penicillins resistant to staphylococcal ß-lactamase**	**Group 4 Extended spectrum penicillins**	**Group 5 Penicillins active against *Pseudomonas aeruginosa***	**Group 6 ß-lactamase resistant penicillins**
Benzylpenicillin		**Isoxazolyl-penicillins**	**Ampicillin**	**Acylureido-penicillins**	
Benethamine penicillin	Azidocillin	Cloxacillin	**Ampicillin condensates**	Apalcillin	Temocillin
Benzathine penicillins	Phenethicillin	Dicloxacillin	Hetacillin	Azlocillin	Foramidocillin
Clemizole penicillins	Phenoxy-methylpenicillin	Flucloxacillin	Metampicillin	Mezlocillin	
Procaine penicillin	Propicillin	Oxacillin	**Ampicillin esters**	Piperacillin	
		Methicillin	Bacampicillin	**Carboxycillins**	
		Nafcillin	Lenampicillin	Carbenicillin	
			Pivampicillin	**Carbenicillin ester**	
			Talampicillin	Carfecillin	
			Amoxycillin	Carindacillin	
			Cyclacillin	Ticarcillin	
			Epicillin		
			Mecillinam		
			Mecillinam ester		
			Pivmecillinam		

2.0. The development of the cephalosporins

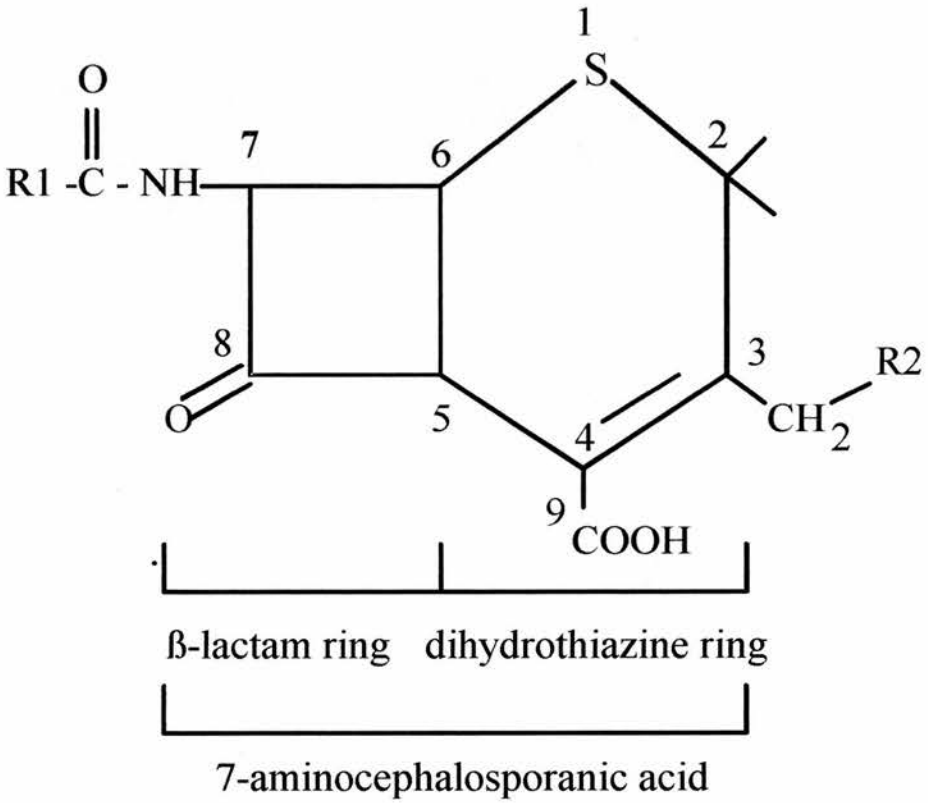
In 1945, Giuseppe Brotzu, Professor of Bacteriology at the University of Cagliari, isolated a strain of *Cephalosporium acremonium* from a local sewage outfall. This mould was found to exhibit antibacterial activity against a wide range of bacterial species. His work is described in an obscure privately published pamphlet called the 'Works of the Institute of Hygiene of Cagliari'. In 1948, Brotzu sent a culture to Howard Florey at Oxford. It was discovered that Brotzu's mould contained at least two antibiotics; a narrow spectrum compound called cephalosporin P and a broad spectrum agent, penicillin N. In 1955, Newton and Abraham published observations on a third antibiotic derived from *Cephalosporium acremonium*, Cephalosporin C [23]. Despite its low antibacterial activity, its high resistance to staphylococcal penicillinase was of great interest.

The cephalosporin nucleus, 7 amino-cephalosporanic acid (7-ACA) has served as the parent structure for many thousands of different cephalosporins. It has the advantage that chemical side groups can be added at two important sites on the molecule (figure 4). Modifications on or near the ß-lactam ring affect antibacterial activity and stability against ß-lactamases, whilst modifications at position C_3 are associated with pharmacokinetic and metabolic parameters [24], allowing therapeutically important modifications to be made.

2.1. Classification of the cephalosporins

The most widely accepted classification of the cephalosporins splits them into three groups; early compounds (the first generation), compounds resistant to ß-lactamases (the second generation), and compounds resistant to ß-lactamases and generally exhibiting enhanced antibacterial activity (the third generation). Lambert and O' Grady have proposed that the cephalosporins be subdivided into 6 groups [25] (table 1).

Figure 4



Chemical structure of cephalosporins and 7-aminocephalosporanic acid

Table 1.

Classification of the cephalosporins

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Cefazaflur	Cefaclor	Cefamandole	Cefepime	Cefixime	Cefoperazone
Cefazolin	Cefadroxil	Cefmetazole	Cefetamet	Ceftibuten	Cefpimizole
Ceforanide	Cefatrizine	Cefodizime	Cefmenoxime		Cefpiramide
Ceftezole	Cefroxadine	Cefonicid	Cefodizime		Cefsulodin
Cephacetrile	Cephalexin	Cefotetan	Cefotaxime		
Cephaloridine	Cephaloglycin	Cefotiam	Cefpirome		
Cephalothin	Cephradine	Cefoxitin	Cefpodoxime		
Cephaprin		Cefuroxime	Ceftazidime		
		Cefbuperazone	Ceftriaxone		
			Ceftizoxime		
			Latamoxef		

After Lambert and O'Grady [25].

Group 1: Parenteral compounds of moderate antimicrobial activity and resistance to staphylococcal ß-lactamase, hydrolysed by a wide variety of enterobacterial ß-lactamases.

Group 2: Oral compounds of moderate antimicrobial activity resistant to staphylococcal ß-lactamase and moderately resistant to some enterobacterial ß-lactamases.

Group 3: Parenteral compounds of moderate antibacterial activity resistant to a wide range of ß-lactamases.

Group 4: Parenteral compounds with potent antimicrobial activity and resistance to a wide range of ß-lactamases.

Group 5: Oral compounds with potent antimicrobial activity and resistance to a wide range of ß-lactamases.

Group 6: Parenteral compounds with moderate activity against enterobacteria, but active against *Pseudomonas aeruginosa* with resistance to a wide range of ß-lactamases.

3.0. Other ß-lactam agents

The discovery of the penicillins and cephalosporins provided the incentive for research into other ß-lactams. Naturally occurring ß-lactam structures have generally been classified by a trivial nomenclature, the name of the compound usually relating to the producing organism and chemical feature of the new compound [26]. A more recent classification, has been delineated on a defined parent ß-lactam skeleton [26], (figure 5).

3.1. Monobactams: Aztreonam is the only monobactam currently in clinical use, they are a family of monocyclic compounds derived from bacteria (*Nocardia* spp.).

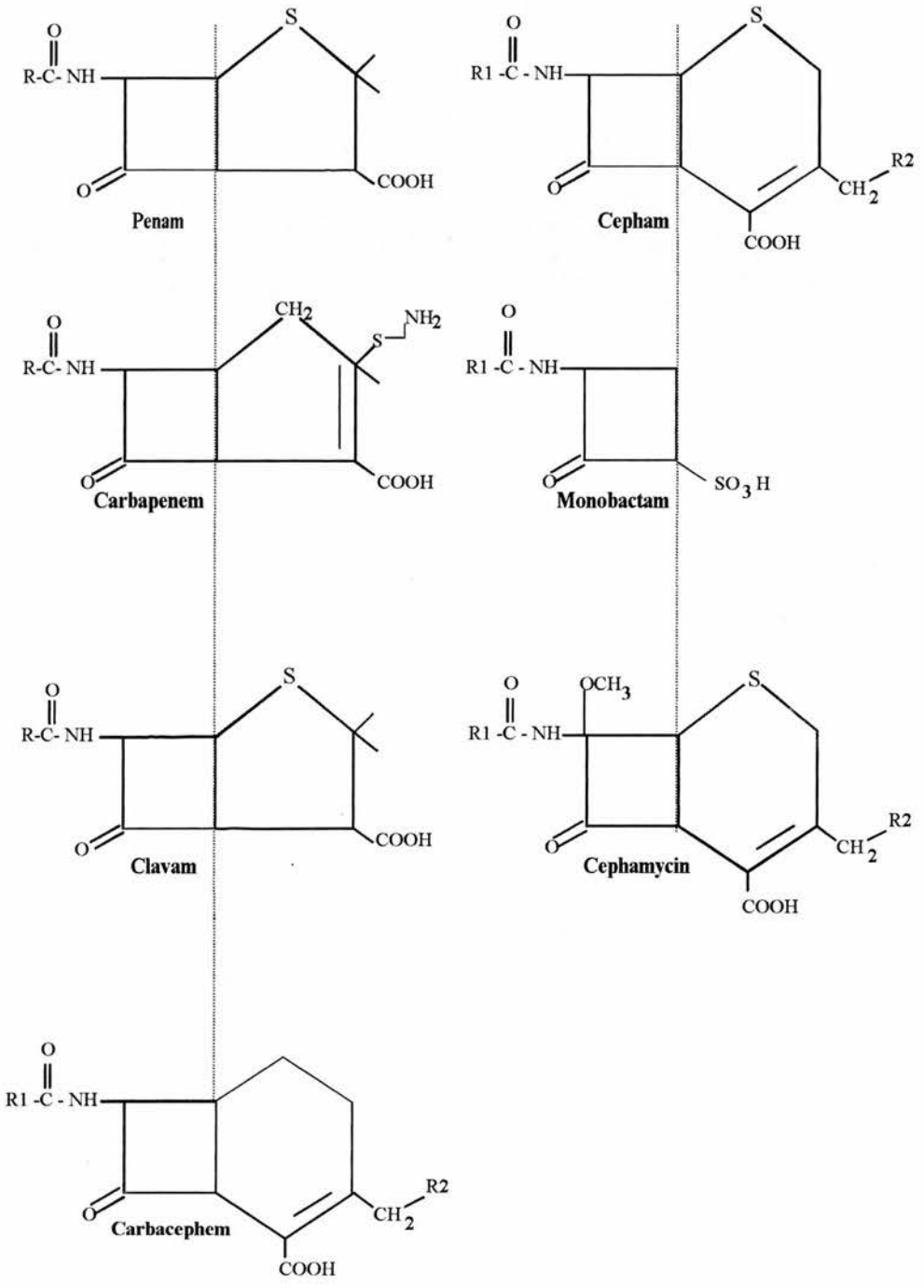
3.2. Cephamycins: Cephamycins are derived from *Streptomyces* spp., and therefore, are very similar in structure to the cephalosporins although different by virtue of a methoxy group present in the seven position of the ß-lactam ring. Removal of the side chain allows modifications, for example cefoxitin.

3.3. Oxacephems: The sulphur atom of the cephem nucleus is replaced with an oxygen atom. An example is latamoxef.

3.4. Penems: The penem nucleus is an unsaturated analogue of the penam ring.

3.5. Carbacephems: The carbacephems are a new class of ß-lactam antibiotics that are similar in structure to the cephalosporins, they differ however, in the substitution of a sulphur atom in the dihydrothiazine ring [27]. Loracarbef is the first of these compounds undergoing clinical development. The development of the carbacephem ring structure may lead to a proliferation of a new series of antibiotics, similar to that experienced with the cephalosporins [27].

Figure 5.



Skeletons of ß-lactam antibiotics. The ß-lactam ring (to the left of the dashed line) is shared by all these compounds.

3.6. Carbapenems: The carbapenems differ from penicillins by the substitution of a carbon atom for a sulphur atom, and by the addition of a double bond to the penicillin nucleus [28]. The first two carbapenems (the olivanic acids and thienamycin) were discovered in the mid 1970s. The olivanic acids were produced by *Streptomyces olivaceus*, but are so unstable that they have not been employed clinically [29]. Imipenem is derived from thienamycin, a natural product of the soil organism *Streptomyces cattleya*. It is the first of the carbapenems available for therapeutic use. The *N*-formimidoyl side chain of imipenem enhances the chemical stability of imipenem. The hydroxyethyl side chain in the α or trans-configuration at C_6 provides remarkable resistance to ß-lactamases [29], and, they exhibit the broadest antibacterial activity of all antibiotics currently available for systemic use in humans [28]. Imipenem is not stable to human dehydropeptidases, it is thus administered with cilastatin, a dehydropeptidase inhibitor. Other compounds, meropenem and biapenem seem sure to join imipenem in the near future. Meropenem contains a unique side chain at C_2 that ensures a broad spectrum of activity. Its main advantage over imipenem is the presence of a methyl group at C_1, that provides stability to human renal dehydropeptidases, therefore, it does not require co-administration with cilastatin.

4.0. ß-lactamase inhibitors

ß-lactamase inhibitors were developed as another approach to find substances that can protect against the problem of ß-lactamases.

4.1. Clavams: Clavam is the parent ring nucleus found in the potent ß-lactamase inhibitor, clavulanic acid. It is a naturally occurring weak antimicrobial agent found initially in strains of *Streptomyces clavuligerus* [30]. The agent primarily acts as a "suicide inhibitor" by forming an irreversible acyl enzyme complex with the ß-lactamase, leading to loss of activity of the enzyme. In clinical use it is combined with amoxycillin, co- amoxyclav (augmentin).

4.2. Sulbactam: Sulbactam is a semisynthetic 6-desaminopenicillin sulphone with weak antibacterial activity [31]. For clinical use, it is combined with ampicillin.

Sulbactam acts as an effective inhibitor of certain plasmid and chromosomally mediated ß-lactamases.

4.3. Tazobactam: Tazobactam is a penicillanic acid sulphone derivative that is structurally related to sulbactam, it is combined with piperacillin (tazocin) for clinical use.

4.4. BRL 42715: The penem BRL 42715 is a potent inhibitor of a broad range of bacterial ß-lactamases with a serine residue at their active site [32] but not against metallo-ß-lactamases [33]. It is more effective against chromosomal cephalosporinases than clavulanate, tazobactam or sulbactam [34]. Unfortunately, problems associated with stability of the compound have determined that it will not be marketed [34].

5.0. Mode of action of ß-lactams

ß-lactam antibiotics inhibit bacterial cell wall synthesis [35]. The bacterial cell wall is constructed from alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues. The final step of cell wall synthesis (transpeptidation) involves the transpeptidase-catalysed cross-linking of peptidoglycan chains.

The targets for ß-lactam antibiotics are the cell wall synthesising enzymes, the penicillin binding proteins (PBPs), carboxypeptidases and transpeptidases [36,37]. PBP's are named for their ability to bind labelled penicillin [38]. They are found in all bacteria, but their number, size, amount and affinity for the ß-lactam antibiotics vary from species to species [38]. PBPs are located on the outer face of the cytoplasmic membrane, in Gram negative bacteria they are pseudoperiplasmic [39].

The action of ß-lactams on the bacterial cell is not the only mechanism by which bacterial cells are inhibited. Indirect inhibition of bacterial growth results from the action of murein hydrolases, which are autolytic enzymes that cause nicks in the cell wall to provide sites for new peptidoglycan synthesis during cell wall enlargement [40]. These enzymes are unaffected during inhibition of cell wall synthesis by ß-lactam antibiotics, and inhibition of PBPs and the unsuppressed activity of murein hydrolyses procure autolysis of the bacterial cell.

6.0. Genetics of antimicrobial resistance

Mayer *et al.* stated, "For microbial evolution to transpire, genetic variability is essential" [41].
There are at least three mechanisms that bacteria utilise to ensure this variability:

Micro-evolutionary change: A point mutation may occur at a nucleotide base pair, altering the target site of the antibiotic.

Macro-evolutionary change: This involves whole scale rearrangement of large sections of the bacterial DNA, at a single event. Such rearrangements may include inversions, duplications, insertions, deletions or transpositions of large sequences of DNA, from one location of the bacterial chromosome to another. These arrangements are usually mediated by insertion sequences or transposons.

A third mechanism is the acquisition of foreign DNA. These sequences are carried by plasmids, bacteriophages or transposons. This mechanism is extremely important as it endows bacteria with an almost limitless capacity to become resistance to antimicrobial agents.

7.0. Mechanisms of bacterial resistance to ß-lactam antibiotics

Resistance to ß-lactam antibiotics may occur at any of the steps involved in the action of the antibiotic [42], and may be achieved through a combination of one or more different mechanisms.

1. Alteration in outer membrane permeability.
2. Alteration in target enzymes (PBPs).
3. Enzymatic degradation of the drug either before or after it enters the bacterial cell.

7.1. Alteration in outer membrane permeability

ß-lactams must first penetrate the bacterial cell wall. This is easy in Gram positive bacteria, which do not possess an outer membrane. The outer wall of Gram negative organisms consists of lipids, proteins, and polysaccharides. The intrinsic susceptibility

of Gram negative bacteria is greatly influenced by the nature of the outer membrane, which can pose a formidable barrier to the entry of ß-lactams [43-45].

The porin proteins of the outer membrane of Gram negative bacteria form channels that facilitate the movement of nutrients and other substances between the extracellular environment and the periplasmic space. Changes in outer membrane permeability limit the inflow of drug [35]. Two major proteins that have been characterised in *Escherichia coli* are OmpF and OmpC [46], although three other porins exist which have also been shown to affect antimicrobial susceptibility; LamD, Phoe E and protein K [47]. OmpF has the highest rate of permeability to the ß-lactams [35]. It has been shown that *E. coli* mutants deficient in OmpF and OmpC are resistant to ß-lactams as a consequence of reduced penetration [46]. Alteration in Gram negative outer membrane permeability plays an important role in resistance to carbapenems. *Pseudomonas aeruginosa* mutants have been described which are specifically resistant to imipenem and meropenem [48,49]. Analysis of these isolates showed markedly diminished amounts of outer membrane protein D2. It has been suggested that protein D2 specifically facilitates the diffusion of carbapenems into the bacterial cell of *Ps. aeruginosa* [50,51]. The imipenem resistant isolates remained susceptible to most other antibiotics reported, suggesting that the protein D2 plays only a small role in the diffusion of other antibiotics into the cell. Livermore [52], demonstrated that imipenem resistance could only be mediated by the loss of the D2 porin when the chromosomal ß-lactamase was also expressed. Carbapenem resistance based on the interplay between the chromosomal ß-lactamase and impermeability, has likewise been described for *Enterobacter cloacae* [53,54] *Enterobacter aerogenes* [55] and *Proteus rettgeri* [54]. Clinical isolates resistant to imipenem have also been reported in *Proteus mirabilis* [56], resulting from the loss of a 24 kDa OMP, no concomitant ß-lactamase was reported in these strains.

7.2. Alteration in target enzymes (PBPs)

Alterations in the binding characteristics of PBPs for ß-lactam compounds may result in the development of resistance [57]. Any given organism can contain between four to eight PBPs, and although only two to four are essential for cell survival, their inhibition can lead to cell lysis, death or growth arrest.

This mechanism is more important in Gram positive bacteria, and the fastidious Gram negative bacteria such as *Haemophilus influenzae and Neisseria gonorrhoeae* [58].

Clinical resistance to ß-lactams in Gram negative bacteria is not commonly associated with altered PBPs [59], although a recent report describes *Acinetobacter baumannii* isolates resistant to imipenem and ampicillin, as a result of decreased binding to all PBPs [60]. PBP associated resistance in clinically important bacteria is shown in table 2.

Table 2

PBP-associated resistance in clinically important bacteria

Organism	Resistant to	PBP (alteration) of species affected
S. aureus	Methicillin, most ß-lactams	2a, 3.
S. epidermidis	Methicillin, most ß-lactams	2a
S. pneumoniae	Ampicillin	1a, 2a, 2b.
Ent. faecalis	Most ß-lactams	1, 3.
E. coli	Mecillinam	2
	Cephalexin	3
Ps. aeruginosa	Piperacillin	3
	Cefsulodin[c]	
H. influenzae	Ampicillin[c]	4
	ß-lactams	3, 4, 5.
A. calcoaceticus	ß-lactams	1,3.
N. gonorrhoeae	ß-lactams	1, 2.
N. meningitidis	ß-lactams	2.
B. fragilis	Cefoxitin	1, 2.

[c] Laboratory strain

From Georgopapadakou [61].

7.3. Enzymatic-mediated antibiotic degradation

The most common cause of resistance to ß-lactam drugs is enzyme mediated antibiotic degradation [35,62,63]. Bacteria encode for three enzymes which can hydrolyse ß-lactams:

1. Esterases
2. Acylases
3. ß-lactamases

By far the most important of these mechanisms is ß-lactamase production [35], the others play a relatively minor role, if any, in antibiotic resistance [64], although esterases are of clinical use when cleaving microbiologically inactive penicillin esters e.g. talampicillin, releasing the active compound.

The first report of a penicillin hydrolysing enzyme was actually discovered before penicillin G was widely used. Abraham and Chain [65] reported that they found an organism, then called *Bacillus coli*, now *Escherichia coli*, that produced an enzyme that inactivated penicillin.

Most early attention focused on staphylococcal ß-lactamases, although by the 1960s, the advent of the broader spectrum ß-lactams, and the success of the anti-staphylococcal drug, methicillin, shifted the focus of attention to the ß-lactamases of Gram negative bacilli.

This thesis will be concerned only with ß-lactamases produced by Gram negative aerobic bacilli.

8.0. ß-lactamases

8.1. The origin of ß-lactamases

It has long been postulated that ß-lactamases might have evolved from the penicillin sensitive D-alanyl-D-alanine-cleaving peptidases (DD-peptidase/PBPs), which are involved in the synthesis of peptidoglycan, and are the target for ß-lactam antibiotics. Kelly *et al.* [66], compared the DD-peptidase from *Streptomyces* R61 with the ß-lactamase from *B. licheniformis* 749/C. Although the two strains were from different species and lacked close homology in their primary structures (although they contained several conserved residues in their active sites) the three dimensional structures of the two enzymes were similar, implying a close relationship between the two. Kelly went on to postulate that ß-lactamases probably evolved from the soil bacteria, like *Streptomyces* spp. Huletsky *et al.* [67], made significant progress in explaining how the Class A ß-lactamases evolved. They constructed a phylogenetic tree, that split the ß-lactamases into Gram negative and Gram positive subgroups and demonstrated that ß-lactamases from Gram positive bacteria probably evolved earlier in evolution than those of the Gram negative bacteria. Kirby [68], elaborating on the work of Huletsky, examined the protein sequences of 18 Class A ß-lactamases and 2 Class C ß-lactamases and constructed a phylogenetic tree employing the DD-peptidase of *Streptomyces* R61 as an outgroup. Kirby's work suggested that all the Class A and C ß-lactamases were evolved from an actinomycete ß-lactamase, that had previously evolved from the DD-peptidase gene.

The previous work does not take the Class B ß-lactamases into consideration, and no work of any note appears to address the evolutionary origin of these ß-lactamases.

8.2. Contribution of ß-lactamase to ß-lactam antibiotic resistance

The level of antibiotic resistance mediated by any ß-lactamase is governed by several variables [41].

1. The efficiency of the ß-lactamase in hydrolysing the antibiotic (V_{max}) and its affinity for the antibiotic (K_m).
2. The amount of ß-lactamase produced.
3. The susceptibility of the target protein (PBP).
4. The rate of entry of the antibiotic into the bacterial cell.

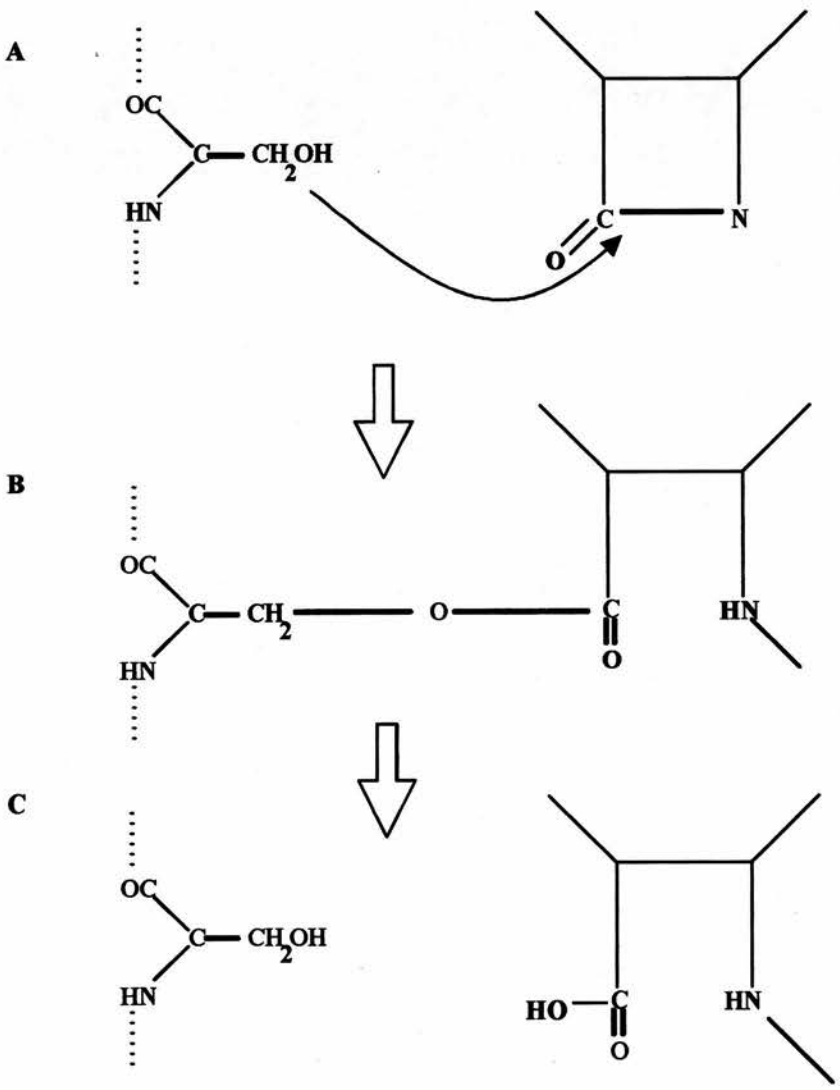
8.3. Action of ß-lactamase

Most ß-lactamases are members of a superfamily of serine proteases [69], which hydrolyse the ß-lactam ring (figure 6). The active sites of most ß-lactamases invariably contain the amino acid residue, serine, and a conserved lysine three residues downstream from the serine [58]. The serine at the enzyme's active site forms a non-covalent complex with the ß-lactam (A). Acylation follows, yielding a covalent ester, in which the carboxyl of the opened ß-lactam ring is linked to the hydroxyl group on the serine side chain (B). The ester is hydrolysed, liberating free enzyme and the hydrolysed substrate (C) [70]. The exception are the Class B ß-lactamases, which require a zinc co-factor for catalytic activity, and which are clearly different from the other enzymes [71]. The Class B ß-lactamases will be discussed in depth in a later section.

9.0. Chromosomal ß-lactamases

Chromosomal ß-lactamases are produced by virtually all Gram negative bacilli [62,72-74], and the nature of ß-lactamase produced is often species-specific [73]. Most of the chromosomally mediated ß-lactamases preferentially hydrolyse cephalosporins, although some organisms also produce a broad spectrum chromosomal ß-lactamase, that hydrolyses a wide range of ß-lactams [73]. All of the chromosomal cephalosporinases have a common evolutionary origin [75], and production is either constitutive or inducible.

Figure 6.



Action of serine ß-lactamase

9.1. Constitutive cephalosporinases

In certain species like *E. coli*, *Shigellae* and *Proteus mirabilis*, the enzyme is produced constitutively, and manufactured in only basal amounts, insufficient to protect the bacteria against drugs, such as ampicillin and cephradine, which are rapidly hydrolysed by the enzymes [76,77]. The gene responsible for production of this chromosomal ß-lactamase is known as *amp*C. Overproduction of ß-lactamase by the *amp*C gene can cause resistance to ß-lactams [78], although it is thought to be a rare occurrence [76]. It has been shown that two separate mutations are required for this high level enzyme production, one increasing a promoter efficiency and the other inactivating an attenuator [79].

9.2. Inducible cephalosporinases

Some species, including *Ps. aeruginosa*, *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., and indole positive Proteus, have inducible expression of chromosomal cephalosporinase. This is simply a transient elevation in ß-lactamase production that occurs when a ß-lactam substrate is present. There are thought to be at least four other genes which are involved in control of ß-lactamase expression; *amp*R, *amp*G, *amp*D, and *amp*E [80] (table 3).

The amount of ß-lactamase induced by a ß-lactam compound depends on the structure of the compound, on its concentration, and the period that the bacteria are exposed to the substrate [81]. Livermore describes four conditions for the interplay of induction and drug inactivation [81].

1. An antibiotic may be labile to the ß-lactamase and be a strong inducer.
2. It may be labile but a weak inducer.
3. It may be stable and a strong inducer.
4. It may be stable and a weak inducer.

Table 3.

Elements involved in the expression of inducible ß-lactamases in Gram negative bacteria

Element	Role or description
*Amp*R	Transcriptional regulation
*Amp*G	Necessary for induction; integral membrane protein responsible for transmission of induction signal
*Amp*D	Down-regulation of ß-lactamase expression; maintenance of repressor form of transcriptional regulation?
*Amp*E	Enhancement of repression; dispensable
PBP2	ß-lactam-interactive protein

Adapted from Bennett & Chopra [80]

The emergence of resistance during therapy with one of the newer generation of ß-lactams was first described in 1978 [82]. This resistance results from selection during therapy of "stably derepressed mutations" (SDM) which produce very high levels of chromosomal ß-lactamase, such mutations may be selected at a frequency of one in 10^6 to one in 10^7 cells [72]. These stably derepressed mutations have seriously eroded the efficacy of the third generation cephalosporins [58].

9.3. Broad-spectrum chromosomal ß-lactamases

Klebsiella spp. produce a number of chromosomal penicillinases, which are normally expressed at low level, however, it is sufficient to confer resistance to various ß-lactam antibiotics [73,83], and accounts for the universal resistance to ampicillin for this genus.

10.0. The plasmid-mediated ß-lactamases

10.1. Broad-spectrum plasmid-mediated ß-lactamases

Transferable ampicillin resistance from *Salmonella typhimurium* to recipient *E. coli* was first reported by Anderson & Datta in 1965 [84]. Subsequently, Datta & Kontomichalou [85] reported the isolation of an ampicillin resistant strain of *E. coli* from a Greek girl called, Temoniera. Both ampicillin resistance and ß-lactamase production could be transferred to a recipient *E. coli* by virtue of a resistance factor (R-factor). The resistance to ampicillin was found to be mediated by a ß-lactamase, subsequently named, TEM-1, and is the most common mechanism of resistance to ß-lactam antibiotics in *E. coli* [86]. Another enzyme, similar in substrate profile to TEM-1, TEM-2, was described by Sykes & Richmond [87]. These enzymes are often carried on mobile genetic elements called transposons, which ensure their efficient dispersal among the Enterobacteriaceae [88]. TEM-1 is known to be commonly carried on Tn*3*. By 1974-76, these enzymes had also spread to *Ps. aeruginosa*, and by 1975 to *H. influenzae* and *N. gonorrhoeae* [62]. In the late 1970s, Petrocheilou [89] described another plasmid mediated enzyme, SHV-1, which was to become the second most common plasmid mediated enzyme after TEM-1. TEM-1, TEM-2 and SHV-1 enzymes are expressed constitutively [76], and are considered to be "broad-spectrum" ß-lactamases that effectively hydrolyse both penicillins and cephalosporins, but not extended-spectrum ß-lactams [90]. Several studies during the 1980s and 1990s in Europe and India have shown that TEM-1, which is found in 70-95% of ampicillin resistant strains of *E. coli* is predominant [91-95]. There have also been a number of other broad-spectrum ß-lactamases reported, however these are rare, and have only been reported on very few or single occasions [96]. It was thought that with the introduction of the third generation cephalosporins in the early 1980's, resistance to these agents mediated by ß-lactamases from Enterobacteriaceae would not develop, however, reports of resistance to these drugs were beginning to emerge, that could not be explained by hyperproduction of chromosomal ß-lactamase or permeability defects [90]. Karen Bush was to quote from Democritus [90]:

"Your victory is in fact your defeat"

10.2. The extended-spectrum ß-lactamases

The explosion of reports of new ß-lactamases is most significant for the extended broad-spectrum ß-lactamases [97], which confer resistance to many of the newer ß-lactam antibiotics. The first of these reports appeared in 1983, Knothe *et al.* [98] described transferable resistance to extended spectrum ß-lactams in strains of *Klebsiella pneumoniae* and *Serratia marcescens*. The resistance was subsequently shown to result from a novel plasmid mediated ß-lactamase, SHV-2 [99]. These ß-lactamases are mostly derived from TEM-1, TEM-2 or SHV-1 (table 4). These mutants differ from their parent enzymes by only one to four amino-acid residues, but have substrate ranges that include not only most penicillins and cephalosporins, but also aztreonam [100]. In a recent report [101], Palzkill and Botstein found that TEM-1 ß-lactamase was very tolerant of amino acid substitutions. They screened 20 different residue positions for amino acid substitutions that increased enzyme activity towards the extended-spectrum cephalosporin, cefotaxime. Substitutions at positions 104, 168, 238 in the TEM-1 ß-lactamase resulted in increased activity toward extended-spectrum cephalosporins. Also, small deletions in the loop containing residues 166-170, increased hydrolytic activity towards the extended-spectrum cephalosporins but drastically reduced the activity towards ampicillin.

A puzzling aspect of the epidemiology of ESBLs is the regularity with which they arise in isolates of *K. pneumoniae*, rather than other members of the Enterobacteriaceae. Katansis & Jacoby [102] demonstrated that neither a difference in the efficiency of expression of ß-lactamase, nor the frequency of ß-lactamase mutations could account for the frequency of ESBLs in *K. pneumoniae* and clearly further study is required.

These ESBLs have been described as "chance mutations", and do not normally appear to have the capacity to spread throughout the general bacterial population [103]. The most frequent reports have emerged from those countries where there has been extensive use of third generation cephalosporins, notably France and North America [104-106]. To date, none of the TEM or SHV derivatives have been found to hydrolyse carbapenems [107].

Table 4.

Molecular basis of extended-spectrum ß-lactamases

	Amino acid at position[1]							
ß-lactamase	**39**	**104**	**164**	**205**	**237**	**238**	**240**	**265**
TEM-1	Gln	Glu	Arg	Gln	Ala	Gly	Glu	Thr
TEM-2	Lys							
TEM-3	Lys	Lys				Ser		
TEM-4		Lys				Ser		Met
TEM-5			Ser		Thr		Lys	
TEM-6		Lys	His					
TEM-7	Lys		Ser					
TEM-8	Lys	Lys	Ser			Ser		
TEM-9		Lys	Ser					Met
TEM-10			Ser				Lys	
TEM-11	Lys		His					
TEM-12			Ser					
TEM-13	Lys							Met
TEM-14	Lys	Lys				Ser		Met
TEM-15		Lys				Ser		
TEM-16	Lys	Lys	His					
TEM-17		Lys						
TEM-18	Lys	Lys						
TEM-19						Ser		
TEM-24			Ser		Thr		Lys	
TEM-26		Lys	Ser					
SHV-1	Gln	Asp	Arg	Arg	Ala	Gly	Glu	Leu
SHV-2						Ser		
SHV-3				Leu		Ser		
SHV-4				Leu		Ser	Lys	
SHV-5						Ser	Lys	
SHV-6								

[1] Amino acid residues are numbered as described by Ambler *et al.* [108]. Abbreviations: Ala, alanine; Arg, arginine; Gln, glutamine; Glu, Glutamic acid; Gly, glycine; Leu, leucine; Lys, lysine; Ser, serine; Thr, threonine; met, methionine. Updated from Jacoby & Medeiros [100]

10.3. TEM-type ß-lactamases resistant to ß-lactam inhibitors

Until recently, all the TEM and SHV enzymes were thought to be very sensitive to the ß-lactamase inhibitors, such as clavulanate, sulbactam and tazobactam. In a recent review, Brumfitt and Hamilton-Miller stated [3]:

"Even the recent development of inhibitors of ß-lactamases (such as clavulanic acid and sulbactam) will probably only alleviate this decline".

They were to be proven correct, with recent reports of TEM enzymes found in clinical isolates with resistance to ß-lactamase inhibitors [109-111].These enzymes have been designated TRC-1 (TEM resistant to clavulanate) and TRI (TEM resistant to ß-lactamase inhibitors). It has been shown that the increased resistance to inhibitors results from a replacement of the methionine residue at position 67 by isoleucine and of the methionine residue at position 180 by threonine [111]. A spontaneous laboratory mutant of OHIO-1 ß-lactamase, an SHV-1 family member, resistant to inhibitors, has also been shown to have methionine at position 67 replaced by an isoleucine residue, and it has been suggested that this residue is the critical residue for resistance to suicide inhibitors for both TEM and SHV [112].

10.4. Plasmid-mediated extended-spectrum ß-lactamases encoded by *amp*C type genes

Recently, another group of extended-spectrum plasmid-mediated ß-lactamases have emerged. The structural gene of these enzymes is related to the *amp*C gene (table 5.). They are all insensitive to clavulanate and have basic isoelectric points. Two of the most extensively studied are MIR-1, which is 90% identical to the nucleotide sequence of the *amp*C gene of *E. cloacae*, and BIL-1, which shows homology with the *amp*C gene of *Citrobacter freundii* [113].

Table 5.

Plasmid-mediated extended spectrum ß-lactamases encoded by *ampC* type genes

ß-lactamase	pI	Structural gene shares homology with *ampC* gene of	Reference
MIR-1	8.4	*Enterobacter cloacae*	[114]
BIL-1	8.8	*Citrobacter freundii*	[113,115]
CMY-1	8.0	Sequence not yet available	[116]
MOX-1	8.9	*Pseudomonas aeruginosa*	[117]

10.5. The future threat of plasmid-mediated ESBLs

Despite a steady increase in the use of more powerful ß-lactams, especially the third generation cephalosporins, over the last few years, TEM-1 and SHV-1 still predominate. This may result from the carriage of ESBLs genes on large plasmids (80 to 300 kb), that do not appear to harbour transposable genetic elements to help in the efficient dissemination of the resistance gene [88]. However, reports of ESBLs **are** becoming increasingly common in the scientific literature, and the continued efficacy of later generation cephalosporins is often held to question. The introduction of the newer oral third generation cephalosporins into community practice may change the current situation, and continued diligence with respect to screening programmes is required. Payne and Amyes [103] remarked that, "....these are still early days and our controlled use of later generation cephalosporins will probably determine whether we can keep these enzymes as a minor inconvenience rather than let them emerge as a major threat".

11.0. The carbapenemases

The carbapenems have the broadest antibacterial spectrum of any of the ß-lactam antibiotics currently available, and are stable against the inducible Class I cephalosporinases, and serine active site ESBLs. These factors will probably guarantee that use of carbapenems will increase in the future. It appears inevitable that this usage will exert selective pressures for resistance to develop. Already there have been reports of ß-lactamase mediated resistance to these drugs. These enzymes may be broadly divided into two groups, by virtue of their mechanism of action:

1. Metallo-ß-lactamases.
2. Serine active site carbapenemases.

11.1. Metallo-ß-lactamases

These enzymes form a small group of ß-lactamases whose catalytic activity appears to be dependant on a zinc ion at the active site, that participates directly in hydrolysis of the ß-lactam ring. They have recently been reviewed by Payne [118], (table 6).

Most of the early reports of metallo-ß-lactamases were from species of little clinical significance, e.g. *Bacillus cereus* [119], *Flavobacterium odoratum* [120], *Legionella gormanii* [121], however, there have been several recent reports of metallo ß-lactamases from more clinically relevant species, e.g. *Aeromonas hydrophila* [122], *Xanthomonas maltophilia* [123,124], *Bacteroides fragilis* [125], and *Ps. aeruginosa* [126]. All these enzymes are chromosomally encoded with the exception of the ß-lactamases from single strains of *Ps. aeruginosa* GN17203 [126] and *B. fragilis* 10-73 [127], which have been found to be carried on conjugative plasmids, that are transferable only within the same species. It is generally thought that most of the chromosomal encoded metallo-ß-lactamases are probably universal within the species [128].

Table. 6

Properties of Metallo-ß-lactamases

Bacterial species	Producer strain	Enzyme name	pI	M_r	Plasmid encoded	Country of isolation	Ref.
B. fragilis	TAL2480	CfiA	4.7	25249		USA	[124]
	TAL 3636						[129]
B. fragilis	GAI30144		4.7	33000		Japan	[130]
B. fragilis	G 237		4.8	26000		Japan	[131]
B. fragilis[a]	BFR 81R	CfiA-type	4.7			France	[132]
B. fragilis	119		4.6			UK	[133]
B. fragilis	QMCN3	CfiA-type		25249		UK	[134]
B. fragilis	QMCN4	CfiA-type		25249		UK	[134]
B. fragilis	GAI30079					Japan	[135]
B. fragilis	KSB1468/90		4.5	31000		Sweden	[136]
B. fragilis	10-73				YES	Japan	[127]
B. distasonis			4.6	160000		Sweden	[137]
B. cereus	569/H/9	II	8.45	24932		UK	[138]
B. cereus	5/B/6	II-type	9.1	22500		UK	[139]
A. hydrophila	19	A-2	8.0	31500		USA	[122]
A. hydrophila	AE036	CphA	8.0	28000		Italy	[140]
F. odoratum	GN14053		5.8	26000		Japan	[120]
L. gormanii	ATCC33297		10.5	25000		Japan	[121]
Ps. aeruginosa	GN 17203		9.0	28000	YES	Japan	[126]
S. marcescens	TN9106	IMP-1	>9.5	30000		Japan	[141]
X. maltophilia	GN12873	L-1	6.9	118000 26000*		Japan	[123,124]

[a] One of four laboratory mutations
*Subunit size

Updated from Payne [118]

A recent report describes a metallo-ß-lactamase (IMP-1) from *S. marcescens* TN9106 [141]. This enzyme was chromosomally encoded with a pI >9.5. Analysis of its amino acid sequence has shown the enzyme to have considerable homology with the metallo-ß-lactamases of *B. cereus*, *B. fragilis* and *A. hydrophila*. The four zinc metal ligands which form the active site of the *B. cereus* ß-lactamase II were shown to be conserved in the amino acid sequence of IMP-1. The G+C content of the bla_{IMP} gene was lower than the species average of *S. marcescens*, suggesting that the imipenem resistance gene had been acquired through transfer of bla_{IMP} from an external source.

To date, there have been reports of metallo-ß-lactamases reported in ten strains of *B. fragilis*, all have similar isoelectric points, and some may be identical. The nucleotide sequence of the TAL3636 (CcrA) [142] ß-lactamase and the TAL 2480 (CfiA) [143] ß-lactamase have been shown to be comparable, and a CcrA gene probe was shown to hybridise with DNA from two of five strains of *B. fragilis* from the UK [134]. A recent report by Podglajen *et al.*, has shown that it is possible to select spontaneous carbapenem-resistant mutants *in vitro*, following exposure to imipenem [132]. The carbapenemase genes in the carbapenem resistant strains selected *in vitro* and *in vivo* were homologous, raising the possibility that the resistance results from a one-step mutation, leading to the expression of a silent carbapenemase gene. Podglajen also demonstrated that DNA from the imipenem resistant strains and some of the imipenem sensitive strains, hybridised with a 720 bp DNA probe from the CfiA gene. This raises an important epidemiological question. Is there a reservoir of *B. fragilis* strains carrying the silent CfiA gene within the *B. fragilis* population? The possibility of treatment failure, resulting from expression of a silent carbapenemase gene, after treatment with a carbapenem, is a worrying concern. The only other species of *Bacteroides* reported to produce a metallo-ß-lactamase is from a strain of *Bacteroides distasonis* [137]. This enzyme appears distinct from the CfiA group of carbapenemases, its isoelectric point is similar (4.8) but the isolate has a lower MIC to imipenem (1 mg/l) than most of the other metallo-ß-lactamases reported from *Bacteroides* spp (>16 mg/l). The enzyme also has a molecular mass, as determined by Sephadex gel filtration, of approximately 4 times that of any of the *B. fragilis* Group carbapenemases. It would be interesting to determine the M_r, employing SDS-PAGE, to ascertain whether the enzyme existed in subunit form, similar to that of the L1 enzyme from *X. maltophilia*.

The metallo-ß-lactamase from *A. hydrophila* AE036 encoded by the CphA gene possesses an unusual spectrum of activity compared with the other metallo-ß-

lactamases identified so far, being active on penems and carbapenems only [144], whereas other metallo-ß-lactamases are known to hydrolyse a variety of ß-lactams, other than carbapenems.

11.2. Mode of action of metallo-ß-lactamases

As previously briefly alluded, the mode of action of metallo-ß-lactamases is different from that of the serine active site ß-lactamases. The original and most extensively studied metallo-ß-lactamase is ß-lactamase II, from *B. cereus*, 569/H/9 and 5/B/6 [138,145,146].

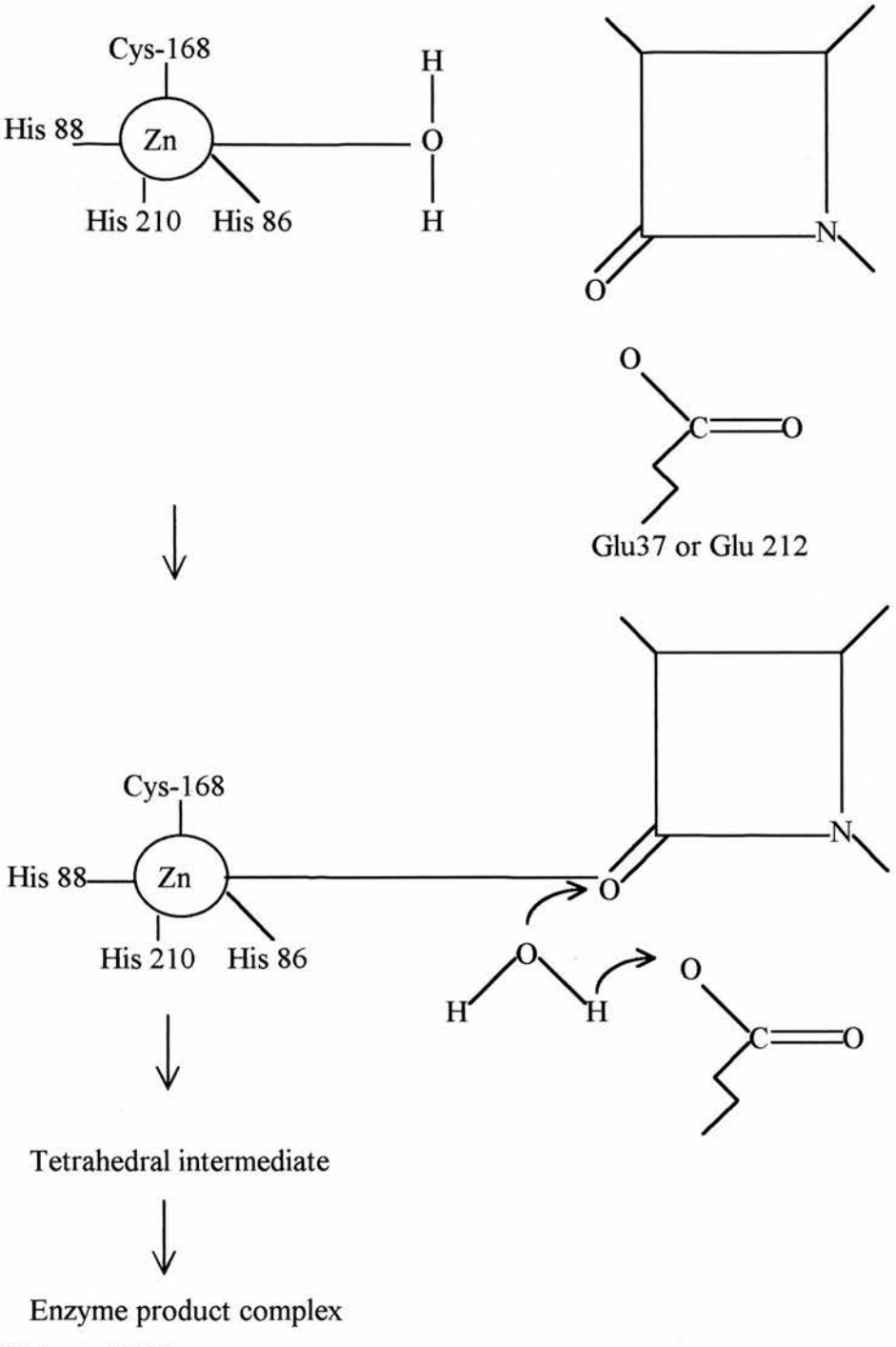
The hypothetical mechanism for ß-lactamase II action was outlined by Little *et al.* [147], and probably reflects the action of all the metallo-ß-lactamases (figure 7). It appears that three histidine residues serve as ligands to the Zn^{++} ion, a water molecule bound to the catalytic Zn^{++}, attacks the ß-lactam carbonyl group, with glutamate-37 acting as a general base, deprotonating the water molecule. The water molecule is then donated to the nitrogen atom of the ß-lactam ring. Subsequent X-ray-crystallographic studies have confirmed that the binding site consists of a Zn^{++} ion surrounded by three histidine residues and one cysteine residue. However, it appears that the distance of glutamic acid-37 is too far from the metal ion to act as a general base, as suggested by Little [147], and that glutamic acid-212, which is considerably closer to the metal ion, may be an alternative candidate.

11.3. Sequence homology between the metallo-ß-lactamases

Table 7 shows the amino acid sequence comparison between the five Class B metallo-ß-lactamases that have been investigated to molecular level.

Figure 7.

Hypothetical outline mechanism for ß-lactamase II action



Tetrahedral intermediate

Enzyme product complex

From Little *et al.*[147]

Table 7.

Amino acid sequence homology between the metallo-ß-lactamases

	Identity (%)		
	B. fragilis CfiA	*B. cereus*	*A. hydrophila*
B. cereus 5/B/6	41.4	-	-
A. hydrophila CphA	34.3	34.9	-
X. maltophilia. L1	22.7	21.4	21.0
S. marcescens IMP 1	35.9	38.9	22.1

Adapted from Felici *et al.*[148], and Osano *et al.*[141]

The sequence comparison of the above enzymes, indicates that the metallo-ß-lactamase L1 from *X. maltophilia* shares little homology with the other enzymes. Collectively, all five enzymes share only 14 strictly conserved residues, but this number is increased to 37 with the removal of the *X. maltophilia* L1 ß-lactamase [148]. It has, therefore been proposed, that the L1 enzyme be placed in a separate subclass of the Class B ß-lactamases [148,149]. Massidda *et al.*[140], also argued that the *A. hydrophila* metallo-ß-lactamase CphA was also sufficiently dissimilar to the original Class B metallo-ß-lactamase, 5/B/6, to warrant its inclusion in a separate molecular subclass from that of *X. maltophilia* L1, although they noted that the sequence homology of the CphA enzyme to the *B. cereus* ß-lactamase II enzyme was more pronounced in the regions known to encompass the active site. Surprisingly, His-86, one of the histidine binding ligands in the *B. cereus* 5/B/6 ß-lactamase was replaced by an aspargine residue in the CphA gene, although this residue has been shown to play only a minor role to the binding of the metal co-factor in ß-lactamase II from *B. cereus* 5/B/6.

11.4. Serine active site carbapenemases

Serine active site carbapenemases, are probably the rarest of all the ß-lactamases. Class I chromosomal cephalosporinases have been shown to slowly hydrolyse imipenem, but resistance to carbapenems is only conferred if the enzyme is produced at high levels with a concomitant decrease in outer membrane permeability as described for overproduction of chromosomal cephalosporinase in *E. cloacae* [53].

A carbapenemase, which when coupled with a permeability factor, confers resistance to a wide range of ß-lactams, including imipenem (16.0 mg/l), has been described in a single strain of *B. distasonis* TAL 7860 [150], although this enzyme has not been shown to be inducible. This strain produces an enzyme with a pI of 6.9. Differences in physicochemical properties and inhibition studies suggest that this ß-lactamase is different from the imipenem-inactivating metallo-ß-lactamases previously described in *B. fragilis*, its V_{max} for imipenem is only 0.2% that for cephalothin. Livermore [151], thought it questionable whether it should appear on a list of carbapenemases at all.

There are only two fully characterised serine carbapenemase reported to date, the NMC-A enzyme from *E. cloacae* NOR-1, described by Nordmann *et al.* [152], and the SME-1 ß-lactamase from *S. marcescens* S6, recently reported by Naas *et al.* [153], although there are partly characterised enzymes from *Serratia* and *Enterobacter* spp, reported only in abstracts, that may belong to this category [154]. The NMC-A ß-lactamase is chromosomally encoded with a molecular mass of 30 kDa and a pI of 6.9. The strain of *E. cloacae* also encodes for a Class I cephalosporinase of pI 9.2. The NMC-A enzyme was not inactivated by the metal ion chelator EDTA, which indicated that it was not zinc dependant. A 1.2 kb DNA fragment of the NMC-A structural gene from a recombinant plasmid failed to hybridise with *amp*C of *E. cloacae*, with other known ß-lactamase genes found commonly in the Enterobacteriaceae (bla_{TEM-1} and bla_{SHV-3} derivatives), and with carbapenemase genes such as those from *X. maltophilia*, *B. cereus*, *B. fragilis* (CfiA), and *A. hydrophila* (CphA). It has however, been shown to share 70% amino acid identity with SME-1, a Class A ß-lactamase [153], which would infer that NMC-A is also a Class A ß-lactamase.

The SME-1 ß-lactamase from *Serratia marcescens* S6 [153], has a pI of 9.7. and a molecular mass of 29.3 kDa. It shares similar hydrolytic properties with NMC-A. SME-1 was initially inferred to be a metallo-ß-lactamase on the basis of data

suggesting inhibition by EDTA [155], however the bla_{sme-1} gene shows no homology with the Class B metallo-ß-lactamases.

A recent report has described imipenem resistance in *E. coli* and the strain was also resistant to most other agents tested. The mechanism of resistance has not yet been ellucidated, although the patient had previously received imipenem and a decrease in permeability is suspected, rather than production of a carbapenemase. We await with interest the outcome of further investigations to determine the cause of the resistance [156].

12.0. ß-lactamase nomenclature

The penicillin destroying activity of extracts from *E. coli*, first described by Abraham and Chain in 1940, was shown to be enzymatic and given the name "penicillinase". In 1965 the Enzyme commission gave penicillinase the official name, when it described the enzyme as "penicillin-amide-ß-lactam-hydrolase", EC 3.5.2.6. With the introduction of new ß-lactams in the 1960s, and the discovery of new ß-lactamases, the term "cephalosporinases" was widely utilised. In 1972, the Enzyme commission introduced "cephalosporinase as EC 3.4.2.8. However, the situation was confused rather that clarified, by the introduction of the term "ß-lactamase I" for penicillinase and "ß-lactamase II" for cephalosporinase. The situation was clarified in 1984. The Enzyme commission finally grouped all ß-lactamases under the one heading, EC 3.5.2.6.

13.0. Classification schemes for ß-lactamases

Classifying the ß-lactamases has proven to be a difficult task [157]. A number of schemes have been proposed. Although most are based on substrate and inhibitor profiles, a molecular classification scheme based on amino acid sequence around the active site has also been proposed. Most of the classification schemes have essentially disregarded the ß-lactamases of Gram positive bacteria, and their attention has been focused on the ß-lactamases of Gram negative bacteria.

13.1. Early classification schemes.

The first early attempts at classification were those by Sawai *et al.* [158] in 1968, and Jack and Richmond [159] in 1970. Sawai listed three types of ß-lactamases on the basis of substrate profile:

Group 1: Inducible cephalosporinases.

Group 2: Cephalosporinases that had properties of penicillinases, or specifically, broad spectrum ß-lactamases identified from *Proteus vulgaris*.

Group 3: Penicillinases.

The classification scheme of Jack and Richmond was essentially similar, but they divided the cephalosporin-hydrolysing enzymes into two categories. They proposed four groups of ß-lactamases.

Group 1: Broad spectrum enzymes.

Group 2: Penicillinases

Group 3: Cephalosporinases having little or no hydrolytic activity against penicillins.

Group 4: Cephalosporinases

Using additional parameters of enzyme inhibition (p-CMB and cloxacillin) and electrophoretic mobility they identified at least eight different ß-lactamase types.

13.2. The Richmond and Sykes classification scheme

In 1973 Richmond and Sykes [160] proposed a classification scheme that is still widely used today. In a recent article by R. C. Moellering Jr. [157], he describes their system as "...the classic scheme".

Richmond and Sykes outlined five broad classes of ß-lactamases from Gram negative bacteria, on the basis of substrate profile and inhibition studies:

Class I: This class comprises enzymes that are predominantly active against cephalosporins. These enzymes are chromosomally mediated by such strains as *E. coli*, *Enterobacter* spp., *Citrobacter* spp., Indole positive Proteus, *Pseudomonas* spp., and *Serratia* spp, and are either produced constitutively or are inducible.
Class I enzymes have been subdivided into four types (table 8).

Table 8.
Classification of chromosomal cephalosporinases

R & S Class and type	Expression	Species	Other names
1a	Inducible	*E. cloacae*	P99
1b	Constitutive	*E. coli*	*Amp*C ß-lactamase
1c	Inducible	*P. vulgaris*	Cefuroximase, cefotaximase
1d	Inducible	*Ps. aeruginosa*	Sabath-Abraham enzyme

Taken from Sanders [161].

Class II: This class comprises enzymes that are predominantly active against penicillins, these are uncommon and have been found in species of *Proteus*.

Class III: This class comprises enzymes that have approximately equal activity against penicillins and cephalosporins but are sensitive to inhibition by cloxacillin and resistant to inhibition by p-CMB. These enzymes are plasmid-mediated and encompass the TEM and SHV ß-lactamases.

Class IV: Class IV enzymes have a substrate profile similar to that of the Class III enzymes but are resistant to inhibition by cloxacillin and sensitive to inhibition by p-CMB. Enzymes of this class include the chromosomally encoded ß-lactamases of *Klebsiella* spp. They are produced constitutively.

Class V: Class V enzymes are all plasmid-mediated, and comprise a heterogeneous group of enzymes. They all exhibit a "penicillinase" profile, including activity against

cloxacillin. They are resistant to p-CMB. Included in this group are the oxacillin-hydrolysing enzymes (OXAs) and the *Pseudomonas* specific enzymes (PSEs).

13.3. The Sykes and Matthews classification scheme

In 1976, Sykes and Matthew expanded on the R & S scheme [74], including, for the first time, the use of isoelectric focusing as a major criterion for differentiating ß-lactamases. They proposed two major groups, Classes A and B, which were further subdivided into three subclasses:

Class A

These enzymes are all chromosomally mediated and are sub classified into:

(a) Penicillinases.

(b) Cephalosporinases.

(c) Broad spectrum ß-lactamases.

Class B

These enzymes are those determined by R-plasmids and are sub classified into:

(a) Enzymes that do not hydrolyse isoxazolyl ß-lactam substrates.

(b) Enzymes that hydrolyse methicillin and isoxazolyl ß-lactam substrates.

(c) Other enzymes specified by R-plasmids.

13.4. Early classification schemes for R-factor-mediated enzymes

The increase of reports of R-plasmid mediated enzymes in the 1970s led to the development of additional schemes to deal specifically with these enzymes. These included the classification schemes of Mitsuhashi *et al.* [162] and Pitton [163]. Mitsuhashi grouped these enzymes into four types:

Type 1: Comprised of the TEM-type enzymes.

Type II: Comprised of the oxacillin-hydrolysing enzymes that also hydrolyse methicillin.

Type III: Comprises the oxacillin-hydrolysing enzymes that do not hydrolyse methicillin.

Type IV: Comprises the carbenicillin hydrolysing enzymes (PSEs).

Pitton subdivided the TEM and SHV enzymes by isoelectric focusing and substrate profile, into Pitton type 1 and 2. In this scheme he refers to SHV as TEM-1 type 2. Neither of these schemes have been widely adopted.

13.5. The Ambler classification scheme

In the second half of the 1970s it began to be possible to classify ß-lactamases according to their molecular structures rather than their enzymatic activities and isoelectric points. In 1980, Richard Ambler proposed a classification scheme based on molecular structure [164]. Ambler originally identified two classes of enzyme, A and B:

Class A: This class was based on the amino acid sequences of *Staphylococcus aureus* PC1, *Bacillus licheniformis* 749/C, *B. cereus* 569/H (ß-lactamase I) and the *E. coli* plasmid mediated ß-lactamase R_{TEM}. All the Class A enzymes have a molecular mass of ~30kDa and share considerable homology. Ambler concluded that their similarity was so great that they had diverged from a single ancestral gene.

In 1991, Ambler [108] introduced a standard numbering scheme for Class A ß-lactamases, by aligning 20 Class A proteins and attaching numbers to the alignment. In his scheme the active site serine residue has been given the number **ABL** 70. The other scheme in general use, is that of Sutcliffe [165]. This numbering system is derived from the natural sequence of TEM-1_{pBR322}. In this scheme the active site serine residue is placed at number 68.

Class B: This class was created for the zinc requiring metallo-ß-lactamase, ß-lactamase II from *Bacillus cereus* 5/B/6. In contrast, it shows no similarity in sequence to the Class A enzymes.

As previously discussed, the other metallo-ß-lactamases (table 7) for which we have amino acid sequences, do not share extensive homology with ß-lactamase II, although the residues around the active site are highly conserved. Sanders [73] went as far to suggest that a new Class, E, be created for the L1 ß-lactamase of *X. maltophilia* [123].

Class C: This class was first recognised in 1981 by Jaurin and Grundström [166]. Confirmation of this group was also shown by Knott-Hunziker *et al.* [167]. Subsequent experiments with DNA probes indicate that this class of enzyme is widely spread through the chromosomes of Gram negative bacteria [75]. These chromosomal cephalosporinases also function with a serine residue at the active site, but are very different in structure to those of the Class A enzymes [73].

Class D: This class was proposed by Huovinen *et al.* [168], who after having deduced the amino acid sequence of the PSE-2 ß-lactamase, found that it shared extensive homology with the OXA-2 ß-lactamase [169]. Neither of these enzymes share structural similarities with TEM or *amp*C ß-lactamases, and were therefore placed in a new Class, D.

13.6. The Bush classification scheme

In 1988, Bush [170], although acknowledging the versatility of the R & S, scheme criticised it on a few salient points. She stated that NaCl should not be used as one of the reference criteria as NaCl-ß-lactamase interaction had not been correlated with any specific structural or mechanistic characteristic. Another criticism was that Gram-positive organisms were not included in the scheme.

Bush, therefore, thought it attractive to consider a revaluation of ß-lactamase classification schemes, though, perhaps from a biochemists viewpoint, she also thought it premature to use the current molecular results to define a grand plan for ß-lactamases [170]. She stated that:

"...it is possible to use much of the information now available to develop a classification system that will be reasonably simple, accurate, and fairly rapid. This scheme must be based upon principles and techniques easily accessible to the clinical laboratory".

In making these comments, Bush had perhaps found an ally. Sykes [64], after his comprehensive review of classification systems at the time, including the scheme of Ambler [164], stated:

"The scheme (Richmond & Sykes) is recommended for continued use on two counts: (1) it has been cited extensively in previous literature, and (2) it can serve as a common reference for future studies on new ß-lactamases".

Clearly Sykes was unhappy at the classification of ß-lactamase by purely molecular means. With this common ground, Bush and Sykes published a much needed set of guidelines [171]. They recognised that the methodology in many studies had led to insufficient and often inaccurate data, with wide variation between different workers, and that these guidelines would bring about a more united approach to the study of ß-lactamases.

The criteria in these guidelines led to the compilation of an updated classification scheme (outlined in table 10), that utilises substrate and inhibitor profiles, in addition to physical data for both plasmid-mediated and chromosomal enzymes [149,172].

In the Bush scheme, four classes of ß-lactamases are described:

Group 1: This class includes many of the inducible chromosomally mediated ß-lactamases from Gram-negative bacteria.

Group 2: This class includes all the important plasmid-mediated ß-lactamases, that are inhibited by clavulanate, sulbactam and tazobactam. All the ESBLs fall into Class 2b'.

Group 3: Group 3 enzymes are metallo-ß-lactamases, not inhibited by the available ß-lactamase inhibitors, and are inhibited by EDTA.

Group 4: Group 4 contains unusual penicillinases not inhibited by clavulanic acid.

The Bush classification scheme is the most versatile and comprehensive to date. It satisfies the biochemist, clinician and to a certain extent, the molecular biologist, and is now the most widely accepted scheme for the classification of ß-lactamases. Although it was acknowledged that all major ß-lactamases would eventually be sequenced, Bush went on to state:

"...the biological activity associated with the enzyme should be emphasised as the most relevant attribute to a novel ß-lactamase".

This statement emphasises the continued requirement for biochemical characterisation of all novel ß-lactamases. It can perhaps be exemplified by the TEM-3 plasmid -

mediated ß-lactamase [173], which differs from TEM-2 by only two amino acid substitutions. Based on molecular structure, they would both be placed in Ambler Class A. This classification may be of worth to the molecular biologist, but clinically it has little value. TEM-3 can hydrolyse, and confer resistance to later generation cephalosporins. This difference in hydrolytic activity has enormous clinical significance.

Table 10.

Bush classification scheme for ß-lactamases

Group	Subtitle	Preferred substrate(s)	Inhibited by*		Representative enzyme(s)
			Clav	EDTA	
1	CEP-N	Cephalosporins	No	No	Chromosomal enzymes from Gram-negative bacteria
2a	PEN-Y	Penicillins	Yes	No	Penicillinases from Gram-positive bacteria
2b	BDS-Y	Cephalosporins, penicillins	Yes	No	TEM-1, TEM-2, SHV-1, CEP-2
2b'	EBS-Y	Cephalosporins, penicillins, cefotaxime	Yes	No	TEM-3, TEM-5, TEM-7, SHV-2, SHV-3
2c	CAR-Y	Penicillins, carbenicillins	Yes	No	PSE-1, PSE-3, PSE-4, CARB-3, CARB-4
2d	CLX-Y	Penicillins, cloxacillins	Yes	No	OXA-1,2,3 OXA-7, PSE-2, *B. fragilis*
2e	CEP-Y	Cephalosporins	Yes	No	*P. vulgaris*, *B. fragilis*
3	MET-N	Variable	No	Yes	*B. cereus* II *X. maltophilia* L1
4	PEN-N	Penicillins	No	?	*Ps. cepacia* *B. fragilis*

* EDTA = ethylenediaminetetraacetic acid; Clav = Clavulanic acid

Adapted from Moellering [157]

13.7. The Payne & Amyes classification scheme for plasmid-mediated extended-spectrum ß-lactamases

Payne and Amyes also acknowledged that it would be a long time before all the ß-lactamase genes would be sequenced, and all the important amino acid changes were known. They recognised a need to classify the growing number of ESBLs, based on biochemical properties. They proposed a classification system for the plasmid-mediated ESBLs, by dividing them into four groups by virtue of their relative hydrolytic efficiencies of cefotaxime and ceftazidime [103]:

Group 1: Those enzymes that hydrolyse cefotaxime and ceftazidime with poor efficiency.

Group 2: Those enzymes that hydrolyse ceftazidime more efficiently than cefotaxime.

Group 3: These enzymes confer a greater resistance to ceftazidime, and have been subdivided into three subgroups:
(a) All TEM derived ß-lactamases.
(b) All SHV derived ß-lactamases.
(c) ß-lactamases of unknown derivation.

Group 4: This group consists of ß-lactamases that confer resistance to all generations of cephalosporins and clavulanic acid.
Because of insufficient data, Payne and Amyes listed five enzymes that do not fit into the classification scheme.

13.8. The Livermore classification scheme for carbapenemases

Although these enzymes are rare, Livermore has proposed a classification scheme [151]. They have been categorised into three groups.

Group 1: Chromosomal enzymes that are ubiquitous in certain species.
Group II: Chromosomal enzymes that are rare.
Group III: Plasmid determined ß-lactamases.

The carbapenem hydrolysing enzymes have already been discussed in some depth, and will not be discussed any further in this section.

14.0. Nosocomial infections

14.1. Definition

A nosocomial infection, is an infection that is not present or incubating when a patient is admitted into hospital [174]. Nosocomial infections may be either endogenous or exogenous [175]. It has been estimated that between 5 and 10% of patients entering U.S. hospitals acquire a nosocomial infection [176]. There are a number of factors that predispose patients to nosocomial infection. Intrinsic or host factors such as age, sex, underlying disease, and immunosuppressive therapies, increase the risk of infection at all sites [174], whereas the importance of extrinsic risk factors varies with the type of infection (table 11).

Table 11

Important extrinsic risk factors for major nosocomial infections

Infection	Risk factor
Urinary tract	Indwelling catheter
	Duration of catheterisation
	Instrumentation
Pneumonia	Endotracheal tube
	Mechanical ventilation
	Thoracoabdominal surgery
	Nasogastric tube
Surgical wound	Preoperative stay
	Preoperative shaving
	Duration of surgery
	Degree of wound contamination
	Presence of foreign body
Primary bacteraemia	Intravascular cannulas
	Duration of cannulation

From Hughes and Jarvis [174]

14.2. Nosocomial Infection - The early years

At the advent of the antibiotic era, organisms such as *S. aureus*, *S. pneumoniae* and ß-haemolytic streptococci were the major causes of hospital infection [177]. The 1960s saw a subsidence in the importance of staphylococci as a result of the discovery of the ß-lactamase stable ß-lactams. They were replaced by the enteric Gram negative bacilli [177]. During the 1980s, there has been a noticeable change in the pattern of hospital infection, partly due to the selective pressures exerted with the use (and abuse) of more powerful broad spectrum antibiotics, such as the third generation cephalosporins and the carbapenems. Recently there has been a resurgence in the Gram positive bacteria in hospital nosocomial infections, with such species as enterococci and multi-resistant staphylococci [177]. Species of glucose non-fermenting Gram negative bacilli, which previously posed few clinical problems, have also begun to emerge as important nosocomial pathogens. These organisms are often multi-resistant to antimicrobials, and have been responsible for outbreaks of hospital infection or colonisation [178]. Two species that have recently been well publicised are *Acinetobacter* spp. and *Xanthomonas maltophilia*.

15.0. Acinetobacter

The genus *Acinetobacter* has emerged as one of the more significant opportunistic pathogens within the hospital environment during the last decade.

These organisms have undergone extensive taxonomic changes, and many different names have been used, such as *Bacterium anitratum*, *Herellea vaginicola* and *Mima polymorpha*. In 1986, the taxonomy of this species was extensively altered by Bouvet and Grimont [179], who delineated 12 species by DNA-DNA-hybridisation. Most *A. baumannii* strains were formerly classified as *A. calcoaceticus* subspp. *anitratus*. This species has been described as the predominant entity in nosocomial infections [178], and is also the species that exhibits the highest level of resistance among the Acinetobacter [180].

15.1. Infections caused by *Acinetobacter baumannii*

Acinetobacter spp are not thought to carry any formal virulence factors, and in general are opportunistic pathogens. Despite this, recent literature is replete with reports of infections caused by *A. baumannii*. The organism has been found to be capable of causing life-threatening infections such as pneumonia, meningitis, endocarditis and septicaemia [181-183].

15.2. Treatment of *Acinetobacter*

Treatment of infections caused by *Acinetobacter* can pose serious problems for the clinician. They are often resistant to multiple antimicrobials. This resistance results from the production of aminoglycoside-modifying enzymes, ß-lactamase production, and/or reduced outer membrane permeability [184-186]. Table 12 shows the results of two recent surveys on the activity of antimicrobial agents against *A. baumannii* [180,186]. Despite wide differences between the susceptibility of different antimicrobials within the two studies, it is apparent that no single agent, with the exception of the carbapenem, imipenem, is effective against all the strains tested. These results clearly show the efficacy of this drug against *A. baumannii*.

15.3. Imipenem resistance in *Acinetobacter baumannii*

In 1992, Neu, stated [187]:

"...Acinetobacter will probably become resistant to these agents (carbapenems) as have many other organisms".

This statement, was in fact, preceded by several years. In 1991, Bergogne-Bérézin and Joly-Guillou, [188] reported two strains of *Acinetobacter* spp. resistant to imipenem, (MICs 4-8 mg/l) that had been isolated as early as 1987, although these levels probably indicate a diminished susceptibility rather than high level resistance. Since then, the same group have isolated 20 more resistant strains. None of these strains exhibited any ß-lactamase activity against imipenem, and a decrease in permeability is suspected as the mechanism of resistance.

Table 12.

Percentage of isolates of *Acinetobacter baumannii* susceptible to various antimicrobials

Antibiotic	% Susceptible [a] (No=54)	% Susceptible [b] (No=95)
Amikacin	72	36
Ampicillin	2	9
Augmentin	16	91
Amp/Sulbactam	52	NT
Aztreonam	2	46
Cefotaxime	31	68
Ceftazidime	55	68
Cefuroxime	NT	1
Ceftriaxone	24	68
Ciprofloxacin	70	6
Gentamicin	33	2
Imipenem	100	100
Piperacillin	33	64

NT, not tested

a, adapted from Vila *et al.*[186]

b, adapted from Seifert *et al.*[180]

There have been other reports of resistance to imipenem, Gehrlein *et al.*, reported strains with MICs of 4 mg/l, as a result of altered PBPs, rather than a decrease in permeability, again there was no evidence of ß-lactamase involvement [60].

The increasing number of reports of imipenem resistance in this species is of great clinical concern as in many cases, imipenem may be the only antibiotic available for treatment. It is perhaps exemplified by an outbreak of imipenem resistant strains of *A. baumannii* (MICs 8-16 mg/l) between 1988-89, in a surgical intensive care unit in Booth Memorial Medical Centre, Flushing, New York. Extensive use of ceftazidime and imipenem resulted in the selection of strains resistant to all classes of antimicrobials, including imipenem. The organism was eradicated with the use of ampicillin/sulbactam (8/4 ratio), although the results of *in vitro* tests indicated that sulbactam was the sole agent responsible for the killing of these organisms. No attempt was made to determine the mechanism of resistance in these strains [189].

16.0. *Xanthomonas maltophilia*

Another organism that is gaining in prominence is *X. maltophilia*. It is a glucose non-fermenting, Gram negative bacillus. *X. maltophilia* is a free-living ubiquitous organism, with a wide geographic distribution. It was originally named *Bacterium bookerii* [190], and was subsequently classified as *Pseudomonas maltophilia* by Hugh and Ryschenkow in 1961 [191]. It was found to be marginally related to *Xanthomonas* species, a family of plant pathogens, and was subsequently included in the genus *Xanthomonas* by Swings *et al.* in 1983 [190]. The significant differences in the two taxa have been outlined on several occasions, and Van Zyl and Steyn [192] proposed that a new genus should be created for *X. maltophilia*, which could be placed together with the genus *Xanthomonas* in a separate natural group. A recent article has proposed the name *Stenotrophomonas* (a unit feeding on few substrates) *maltophilia* [193]. In this thesis the name, *Xanthomonas maltophilia*, will be used.

16.1. Infections caused by *X. maltophilia*

This organism is increasingly recognised as an important opportunistic pathogen. Severe infections, including septicaemia, peritonitis, meningitis and endocarditis, may be caused by this organism in seriously ill and immunocompromised patients [194-197].

16.2. Mechanisms of resistance in *X. maltophilia*

The emergence of *X. maltophilia* as a pathogen has been attributed to selective pressure by the use of broad spectrum antibiotics [198]. Extensive use of imipenem is a primary cause of this selection. It is one of the few clinically relevant Gram negative bacilli that is resistant to this antimicrobial. Recent studies have shown that the species is commonly resistant to a wide range of ß-lactams as well as other classes of antimicrobials [199-201]. This resistance has been attributed to the interplay between outer membrane impermeability [202] and the production of two potent chromosomal ß-lactamases. L1, a metallo-ß-lactamase [123] and L2, described as an unusual cephalosporinase [203] which between them hydrolyse virtually the entire spectra of ß-lactams. It has been assumed that all strains produce the L1 and L2 enzymes. However, two recent studies have shown an unexpected heterogenicity amongst ß-lactamases produced by the species [204,205]. Both studies reported that there were several ß-lactamases which could be differentiated by their isoelectric points. In the study by Cullmann [205], none of the strains examined produced more than one ß-lactamase.

16.3. Treatment of *X. maltophilia* infections

The control of *X. maltophilia* infections remains a particular problem to clinicians. A recent study by Garcia-Rodriguez *et al.* of *in vitro* susceptibilities to 42 strains of *X. maltophilia*, found that no single antibiotic was effective for all strains tested [200]. ß-lactams fared particularly badly, with the exception of ceftazidime (30.9% resistance). The trimethoprim-sulphamethoxazole combination appeared to be the most efficacious, with 74% of strains sensitive. Another survey by Elting and Bodey [195] reported similar findings, with 89% of strains sensitive to these drugs. Other classes of antibiotics also fare less well in the treatment of *X. maltophilia*, although ciprofloxacin appears reasonably effective. In the survey of Garcia-Rodriguez *et al.*, 40.5% of strains were sensitive, compared with 92% of strains sensitive in the Elting and Bodey study [195].

CHAPTER 2 Materials and Methods

1.0. Bacterial Strains

All bacterial strains investigated in this thesis were collected from the blood of patients in the Royal Infirmary of Edinburgh (RIE) during the period 1980-1991.

All strains were stored initially as lyophilised cultures, and reconstituted by adding 200µl of sterile distilled water, allowed to stand for 15 minutes, plated out onto both Colombia agar supplemented with 5% horse blood and MacConkey agar and incubated overnight at 37°C in air. The purity and integrity of the strains was verified at this point. The strains were then sub-cultured onto nutrient agar slopes and incubated overnight at 37°C, before storage at room temperature in darkness, until required.

Strains that were subjected to further investigations were also stored at -70°C in nutrient broth with sterile glycerol to a final concentration of 10% v/v.

All strains were identified at the time of isolation by either the API20E or API20NE system (API System, S. A. France). This work was performed by staff of the blood culture laboratory, Clinical Bacteriology, Dept. of Medical Microbiology, Edinburgh University.

Details of individual strains are held on a separate document, in the Clinical Bacteriology laboratory, Dept. of Medical Microbiology, Edinburgh University.

Details of the cefuroxime-resistant strains and their ß-lactamase content are listed in the appendix.

Tables 11-13, list the standard bacterial strains, standard bacterial plasmids and the standard ß-lactamase producing strains used in this thesis.

Table 11.

Standard bacterial strains

Strain	Genotype	Source*	Original Reference
NCTC 6571 *S. aureus*		CPHL	
NCTC 10662 *Ps. aeruginosa*		CPHL	
NCTC *E.coli* 10418		CPHL	
Ps. aeruginosa PAO8		SGBA1297	
E. coli C600		SGBA1125	
E. coli K-12 J62-2	*pro*$^-$, *his*$^-$, *trp*$^-$, *lac*$^-$, Rif^R	DP55	[206]

Table 12.

Standard Bacterial plasmids

Plasmid	Markers	Size (kb)	Source*	Original Reference
R1	Ap Cm Km Su *inc*FII	90	DP 6	[207]
RP4	Ap Km Tc *inc*P-1	52	DP42	[208]
R1010-6	Ap (*tra*$^-$) *inc*N	54	DP38	[89]

* CPHL, Central Public Health Laboratories, Colindale, London; DP denotes Dr D. Payne culture collection; SGBA denotes Professor S.G.B. Amyes culture collection.

Table 13.

Standard ß-lactamase producing strains

Bacterial strain	ß-lactamase produced (pI)	Plasmid	Source*	Original reference
E. coli K12 J62-2	TEM-1 (5.4)	R1	DP6	[207]
E. coli 7604	TLE-1 (5.55)	pMG204b	DP37	[209]
E. coli J53-2 2141E	SHV-1 (7.6)	R1010-6	DP38	[89]
E.coli J53-2	OXA-1 (7.4)	R455	SGBA1336	[210]
E.coli J53-2	OXA-4 (7.5)	pMG204	SGBA1337	[210]

*DP denotes Dr D. Payne culture collection
SGBA denotes Professor S.G.B. Amyes culture collection

1.1. Information storage and retrieval of survey strains

All blood cultures isolates collected were suffixed with a number ranging from 1-13 indicating the year of isolation (1=1980, 2=1981 etc.), and the letter "B".

Data concerning blood culture isolates from Edinburgh Royal infirmary was stored in the database program, dBASE III+ (Ashton-Tate, Milton Keynes). All information regarding aerobic Gram negative bacilli was retrieved with this system.

2.0. Materials

2.1. Media

Unless otherwise stated, all growth media used was sterilised by autoclaving at 15 lbs/in^2 at 121^{o}C for 15 minutes.

2.1.1. Complex media

The following complex media were used: Isosensitest Agar (ISTA) (CM471), Nutrient broth No 2 (CM 67), MacConkey agar (CM7), Mueller Hinton agar (MHA) (CM 337), Diagnostic Sensitivity Test Agar (DSTA) (CM261), Colombia Agar (CM331) with 5% added horse blood (Equine Bovine Products, Bonnybridge, Stirlingshire). All materials were supplied by Oxoid, Unipath (Basingstoke, Hants.) unless otherwise stated.

2.1.2. Minimal Media (DM agar)

Minimal media was prepared as described by Davis and Mingioli [211] (table 14). Fifty ml of double strength DM was then supplemented with the appropriate amount of the required amino acid stock solution to achieve the final concentrations shown in table 15. After the appropriate antibiotics were added, 2.5ml of a 20% solution of D-glucose was added and the volume made up to 60ml with sterile distilled water (SDW). This solution was mixed and added to 40ml of molten Bacteriological Agar No 1 (1g of agar/40 ml of SDW). The solution was mixed well and 20ml of the agar solution added to each plate.

A single strength salts medium was prepared by dilution of double strength DM with an equal volume of SDW. This was used for dilution of bacterial cell suspensions.

Table 14.

Preparation of double strength Davis and Mingioli basal medium

Ingredient	Chemical formula	Quantity dissolved in 1L (g)
di-Potassium hydrogen phosphate	K_2HPO_4	14.0
Potassium *di*-hydrogen phosphate	KH_2PO_4	6.0
tri-Sodium citrate	$Na_3C_6H_5O_7$	1.0
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	0.2
Ammonium sulphate	$(NH_4)_2SO_4$	2.0

Table 15.
Amino acid solutions

Solution	Concentration of stock solution (g/l)	Final conc. (mg/l)	Method of sterilisation
L-histidine (BDH)	5.0	50	Steaming/30min
L-methionine (BDH)	5.0	50	Steaming/30min
L-proline (BDH)	5.0	50	Steaming/30min
L-tryptophan (Sigma)	2.0	50	Steaming/30min

2.1.3. Buffers

All buffer solutions used in this thesis were prepared as described in Data for Biomedical Research (Oxford University Press, 1974), unless otherwise stated.

2.1.4. Antimicrobial agents

The antimicrobial agents used and their suppliers are listed in Table 16.

Antibiotic discs

All antibiotic discs were obtained from Mast Laboratories Ltd, Liverpool, UK.

3.0. Methods

3.1. Antibacterial susceptibility testing

3.1.1 Minimum Inhibitory concentrations (MICs)

All minimum inhibitory concentrations were performed on ISTA or MHA agar, unless otherwise stated, following the guidelines established by the British Society for Antimicrobial Chemotherapy (BSAC) [212]. Bacterial strains to be tested were grown overnight in nutrient broth and diluted in single strength DM to give approximately 10^6 cfu/ml. A 1µl volume of this suspension was inoculated onto the surface of the plate, employing a Denley multipoint inoculator (Denley, Surrey) to

give a final inoculum of 10^4 cfu/spot. *E. coli* NCTC 10418, *Ps. aeruginosa* NCTC 10662 and *S. aureus* 6571 were incorporated as control organisms. A plate with no added antibiotic was established as a positive control. The plates were incubated in air for 18 hours at 37°C. The MIC was determined as the first dilution to inhibit all visible growth.

Table 16.

Antimicrobial agents

Antimicrobial Agent	Supplier
Ampicillin	Sigma Chemical Co. Ltd
Aztreonam	E. R. Squibb and Sons
Benzylpenicillin	Sigma Chemical Co. Ltd
BRL 42715	SmithKline Beecham Pharmaceuticals
Carbenicillin	SmithKline Beecham Pharmaceuticals
Cefotaxime	Roussel Laboratories Ltd
Ceftazidime	Glaxo Group Research Ltd
Cefuroxime	Sigma Chemical Co. Ltd
Cephaloradine	Glaxo Group Research Ltd
Ciprofloxacin	Bayer UK Ltd
Clavulanic acid	SmithKline Beecham Pharmaceuticals
Gentamicin	Nicholas Laboratories
Imipenem	Merck Sharp and Dohme Ltd
Meropenem	Zeneca Pharmaceuticals
Netilmicin	Schering Plough Ltd
Nitrocephin	Glaxo Group Research Ltd
Rifampicin	Le Petite
Tobramycin	Lilly

3.1.2. Disc sensitivity testing

Disc sensitivity testing was performed and interpreted as sensitive, intermediate or resistant by Stoke's method as described by Holt and Brown [213].

3.2. Conjugation experiments

The conjugation experiments were performed as described previously by Amyes and Gould [214]. Broth cultures of donor and recipient strains were incubated overnight in nutrient broth, then 4.5 ml of fresh pre-warmed, nutrient broth was inoculated with 0.1 ml of the donor strain and 1.0 ml of the recipient strain and gently mixed. This mixture was then incubated at either 30°C or 37°C for 6 hours. The bacterial cells were harvested, and resuspended in 5.6 ml of single-strength DM and a series of ten-fold dilutions prepared. A 0.1ml volume of each dilution was spread onto minimal media plates containing the appropriate auxotrophic supplements and selective antibiotics. The plates were then incubated for 48 hours at 37°C.

All colonies selected were verified by checking the auxotrophic requirements and resistance markers.

***E. coli* recipients:**

To select ampicillin or imipenem resistant *E. coli* K12 J62-2 transconjugants, minimal media containing the auxotrophic requirements of this strain, histidine (50mg/l), tryptophan (50mg/l) and proline (50mg/l), plus 25 mg/l rifampicin and 50 mg/l ampicillin or 1 mg/l imipenem, as appropriate for selection, was used.

***Ps. aeruginosa* recipients:**

Ps.aeruginosa PAO8 was mutated to rifampicin resistance by inoculating an ISTA plate containing 25mg/l rifampicin with a 100µl aliquot of an overnight nutrient broth culture grown at 37°C, and incubated at 37°C for 48 hours. Mutant colonies were selected and mated with *A. baumannii* 6B92. Transconjugants were selected on ISTA plates containing 1mg/l imipenem and 25mg/l rifampicin.

***Acinetobacter* recipients:**

A clinical isolate of *A. baumannii* (lab no 5255/3/91) resistant to gentamicin (MIC 128mg/l) was mated with *A. baumannii* 6B92 and transconjugants selected with gentamicin (10mg/l) and imipenem (1mg/l) on ISTA.

3.2.1. Plasmid mobilisation

A 0.1 ml volume of an overnight broth culture of the donor strain harbouring the *inc*P-1 plasmid, RP4, was added to 1ml of recipient culture in 4.5ml of pre-warmed nutrient broth. The mixture was incubated at 37°C for 18 hours. A 0.1ml volume of this mixture was added to 4.5 ml pre-warmed nutrient broth along with 1 ml of an overnight broth culture of recipient *E. coli* K12 J62-2. The mixture was incubated for 6 hours at 37°C. The selection procedure was performed as described above.

3.2.2. Transformation of plasmid DNA from *A. baumannii* 6B92 to *Escherichia coli* C600

3.2.2a. Preparation of competent *E. coli* C600 cells

Five ml of an overnight nutrient broth culture of *E. coli* C600 was grown with shaking at 37°C and added to 200ml of prewarmed nutrient broth. The culture was grown with shaking at 37°C until the OD540 (Spectronic 20, Bausch and Lomb, UK) was between 0.3 and 0.4 so that the cells were in the mid-exponential phase of growth. The cells were immediately cooled in an ice-bath and harvested by centrifugation (10000 x *g*, 8 minutes, 4°C, Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments, Stevenage, Herts.). The cells were resuspended in 10ml of ice-cold 0.1M $MgCl_2$ and incubated on ice for 20 minutes, then harvested by centrifugation as before. The cells were then resuspended in 10ml of ice-cold $CaCl_2$ and dispensed into 200µl aliquots into pre-chilled eppendorf tubes for 2-4 hours prior to use.

3.2.2b. Transformation of *E. coli* C600

The plasmid preparation from *A. baumannii* 6B92 (prepared as described in section 3.8.1.) was dissolved in 100µl of TE buffer (10mM Tris-acetic acid (pH8.0), 2mM EDTA). Five µl of this mixture was added to one tube of competent cells and 50µl to another and left on ice for 30 minutes. The tubes were then transferred to a 37°C waterbath for 10 minutes and then 200µl of nutrient broth added to each tube. The cells were then incubated for 1 hour at 37°C and spread onto ISTA plates containing 1mg/l of imipenem. The plates were incubated at 37°C for 48 hours.

3.3. ß-lactamase preparation

3.3.1. Small scale preparation of crude cell free extracts

The strain under test was inoculated onto the surface of a nutrient agar slope and incubated overnight at 37°C. Cells were then washed off the agar surface with 1.0ml of 50 mM sodium phosphate (pH 7.0.) buffer and transferred to a separate container. The cells were then disrupted by ultrasonication (8µM, 30s, MSE Soniprep 150, MSE Instruments, Crawley). The cell lysate was cleared by centrifugation (11600 x *g*, 10 min, 4°C, MSE Microcentaur centrifuge). The resultant cell free supernatant was stored at -20°C until required [264].

3.3.2. Large scale preparation of crude cell free extracts

The bacterial strain under investigation was grown with shaking (200 osc/min) at 37°C in 9ml of nutrient broth. This was used to inoculate a 1L culture of pre-warmed nutrient broth. The cells were grown with shaking at 37°C overnight and harvested by centrifugation at 6000 x *g* for 15 min at 4°C (Sorvall RC-5B). The pellet was resuspended and washed in 20ml of 50mM sodium phosphate buffer (pH 7.0), unless otherwise stated, and recentrifuged at 2500 x *g* for 15 minutes (Heraeus Christ, Bactifuge). The pellet was resuspended in 4ml of the same buffer and disrupted by ultrasonication with constant cooling (8µM, 1 min x 3, Soniprep). The cell debris was removed by centrifugation at 11600 x *g*, 10min, at 4°C. The crude ß-lactamase preparation (supernatant) was stored at -20°C until required.

3.3.3. Induction of small scale ß-lactamase preparations from *X. maltophilia*

A 20ml volume of Mueller Hinton broth (MHB) was inoculated with the strain under test and grown overnight at 37°C with continuous shaking. This was added to 180ml of pre-warmed MHB and incubated for a further 90 minutes. For induction, imipenem or cefotaxime was then added at ¼ the MIC. After incubation for a further 3 hours the cells were harvested, washed in 50 mM Tris Cl buffer (pH 8.0), and the ß-lactamase released as previously described for small-scale preparation of crude enzyme extract.

3.3.4. Induction of large-scale ß-lactamase preparations from *X. maltophilia*

The technique was essentially that described for small-scale ß-lactamase induction of ß-lactamase, except a five-fold increase in the volume of broth was used. The ß-lactamase enzymes were extracted as previously described for large-scale preparation of crude enzyme extracts.

3.4. Assessment of ß-lactamase activity of ß-lactamase preparations (Nitrocephin spot test)

The time in seconds for a 30µl volume of the ß-lactamase preparation to change 100µl of a solution of nitrocephin (50mg/l) from yellow to red was taken as an indication of the ß-lactamase activity of the enzyme preparation.

3.5. Analytical isoelectric focusing

ß-lactamases were identified by analytical IEF as described by Matthew *et al.*[215]. The ß-lactamase extracts were focused on a thin layer polyacrylamide gel containing broad range ampholines (pH 3.5-10.6), or a mixture of broad range and narrow range (pH 4-6) carrier ampholines (ratio 1:1). The composition of the gel is shown in table 17.

Focusing was usually performed on an apparatus comprised of two glass plates 1mm apart. One plate was siliconised with Sigmacote siliconising solution, whilst the other was coated with a binding solution (0.5%^{W}/v gelatine and 0.5%^{W}/v chromium potassium sulphate dodecahydrate (both Aldrich Chemical Co. Ltd, Gillingham, Dorset.) dissolved in sterile distilled water, and allowed to dry in air for 1 hour prior to use) to promote adhesion of the gel to the glass plates prior to use.

Occasionally focusing was performed with an LKB 2217 Ultraphor Electrofocusing unit (Pharmacia, Uppsala, Sweden), with a pre-poured polyacrylamide gel containing broad range ampholines, pH range 3.5-9.5 (PAGplate, Pharmacia).

Samples of ß-lactamase preparations were applied close to the anode on the surface of the gel. The time taken (in secs) for the nitrocephin spot test to show a positive reaction was equivalent to the amount of ß-lactamase preparation (in µl) applied to the gel. A maximum of 60µl of enzyme preparation was added to any one lane.

3.5.1. Running conditions

Isoelectric focusing was performed at 4°C at 1W (constant), 550V (limiting) and 20mA (limiting) for 18 hours. Five µl of wide-range isoelectric point markers, pI range 4.7-10.6 (BDH, Poole, England) were loaded onto the gel in order to quantify the gradient.

Table 17.

Composition of a conventional IEF gel

Material	Supplier	Volume (ml)	Final concentration
5% V/v TEMED* in distilled water	Sigma	0.2	0.25 mg/l
40% W/v ampholines pH 3.5-10 pH 3.5-10/pH 4-6 (Ratio 1:1)	Sigma	2.0	2.0%W/v
Acrylamide (100g) plus methylene bisacrylamide (2.7g) in 300 ml distilled water	BDH	9.0	acrylamide: 75g/l bisacrylamide: 2g/l
Distilled water		25	
Riboflavin (20mg/l)	Sigma	4.0	2mg/l

*TEMED: Tetramethyl-ethylenediamine

3.5.2. Staining

ß-lactamase activity was visualised by repeatedly overlaying the surface of the gel with filter paper (Whatman No 54, BDH), previously dipped in nitrocephin solution (500mg/l). ß-lactamase activity appeared red on a yellow background.

3.5.3 Photography

Photographs of focused ß-lactamases were taken with a Polaroid camera (settings; B4, F8) with a green filter system.

3.6. Partial Purification of ß-lactamases by Sephadex gel filtration

3.6.1. Preparation of Sephadex G-75 and G-150 gel filtration columns

The media (Sephadex G-75 or G-150, Pharmacia) was swollen with the appropriate volume of buffer (50mM sodium phosphate buffer (pH7.0) for G-75, 50mM Tris Cl (pH 8.0) plus 0.1 mM zinc sulphate (Sigma) for G-150) at 100^{o}C for 1 hour. The slurry was allowed to cool to 4^{o}C and poured into a 2cm^{2} x 90cm acrylic column (Amicon Ltd, Stonehouse, Glos.) until packing was complete. When fully packed the top was connected and a flow rate of 2ml/10min established in an ascending direction with an LKB 10200 peristaltic pump (Pharmacia). The gel was allowed to equilibrate for 48 hours with the appropriate buffer before use. After each run, the column was flushed with two volumes of buffer (180ml).

3.6.2. Calibration of Sephadex G-75 and G-150 gel filtration columns

The Sephadex columns were calibrated according to the method of Andrews [216]. Three proteins of known molecular weight were applied to the column (see table 18) in a 1ml aliquot and eluted at a rate of 2ml/10mins in the appropriate buffer.

After application of the standard proteins the eluted fractions were collected in 2ml volumes with an LKB 2070 Ultrorac II fraction collector (Pharmacia). The elution of

Table 18.

Protein standards (10mg in 1 ml buffer) applied to Sephadex column

Column	Protein Standards (Sigma)	Molecular size (daltons)
Sephadex G-75	ovalbumin	45000
	chymotrypsinogen	25500
	cytochrome-C	12384
Sephadex G-150	alcohol dehydrogenase	150000
	bovine serum albumin	66000
	cytochrome-C	12384

the standard proteins was determined by measuring absorbance (λ = 280nm) of the fractions with a Perkin-Elmer UV/Vis Lambda 2 Spectrophotometer (Beaconsfield, Bucks.). The position of the protein markers was then established by plotting the absorbance of each fraction against the fraction number. A standard curve of M_r versus fraction number could then be drawn.

The void volume of the column was established by the application of a 1ml volume in buffer of 10mg/l blue dextran (M_r >2000kDa) (Sigma), to the column.

3.6.3. Determination of the M_r of ß-lactamases

One ml of the crude large-scale ß-lactamase preparation, prepared as described previously, was applied to the column. The flow rate of the column and the settings of the fraction collector were those employed for the calibration procedure.

ß-lactamase activity in any of the eluted fractions was established primarily with the nitrocephin spot test described previously. These fractions were then assayed by the spectrophometric method of O'Callaghan *et al.*[217].

Fractions containing significant activity were pooled and used for further spectrophotometric assays described in the following section and also to load IEF gels.

3.7. Kinetic studies on ß-lactamases

3.7.1. Measurement of ß-lactamase activity

All spectrophotometric studies of ß-lactamases were performed on either a Perkin-Elmer UV/Vis Lambda 2 Spectrophotometer or a Perkin-Elmer UV/Vis 554 Spectrophotometer. All assays were performed at 37°C in either 50mM sodium phosphate buffer (pH7.0) or 50mM Tris Cl (pH8.0). Substrate solutions were freshly prepared in 50mM sodium phosphate buffer (pH7.0). Penicillin substrates were prepared at 10^{-2}M, monobactams, cephalosporins and carbapenems were prepared at 10^{-3}M. Nitrocephin was prepared at 10^{-4}M.

Assays were performed at the wavelength (λ) of maximal absorbance for the ß-lactam ring of each drug (Table 19).

Table 19.

Maximal wavelengths for the measurement of ß-lactam hydrolysis

ß-lactam compound	Molecular weight (M_r)	Wavelength (λ_{max})
Ampicillin	371.4	238nm
Azlocillin	483.5	240nm
Aztreonam	435	318nm
Carbenicillin	422.4	236nm
Cefuroxime	446.4	260nm
Cefotaxime	477.4	265nm
Ceftazidime	636.6	260nm
Cephaloridine	415.5	255nm
Imipenem	316.4	299nm
Meropenem	437.5	300nm
Nitrocephin	516	384nm
Benzyl-Penicillin	356.4	238nm

A 0.1ml aliquot of ß-lactamase preparation was added to a test cuvette containing 0.3ml of substrate and 2.6ml of the appropriate buffer. Control cuvettes were

established with no added substrate. Monitoring of the decrease in absorbance over time (in minutes) was begun immediately. The initial linear part of the reaction curve was used to obtain the rate of hydrolysis.

This was expressed as μM of substrate hydrolysed per minute, per millilitre of enzyme solution (R) [217].

$$\mathbf{R} = \frac{\Delta\mathbf{A} \times \boldsymbol{n} \times \boldsymbol{d}}{\mathbf{A_0} \times \boldsymbol{t}}$$

where: $\mathbf{R}$ = μM of substrate hydrolysed/ minute/ ml of enzyme solution

$\Delta\mathbf{A}$ = change in absorbance

$\boldsymbol{n}$ = μM substrate in cuvette (0.3 for cephalosporins, 3.0 for penicillins, 0.03 for nitrocephin)

$\boldsymbol{d}$ = enzyme dilution adjusted to 1ml

$\mathbf{A_0}$ = absorbance of intact substrate

$\boldsymbol{t}$ = time (in minutes)

3.7.2. Specific activities

All measurements were performed on a Perkin-Elmer UV/Vis Lambda 2 Spectrophotometer. Nitrocephin (10^{-4}M) was employed as the test substrate. The rate of hydrolysis was calculated as described above.

Protein concentration was calculated as described by Waddell [218]. The ß-lactamase preparation was diluted in distilled water 1:100, prior to measurement of absorbance at 215nm and 225nm. The difference in absorbance between the two measurements was multiplied by the correction factor for the spectrophotometer (165). The specific activity of the ß-lactamase was expressed as nanomoles of nitrocephin hydrolysed min/ mg of protein.

3.7.3 Determination of Michaelis Menton kinetics

Values of V_{max} and K_m were derived by linear regression analysis of Lineweaver-Burk plots of initial velocity data at different substrate concentrations [219]. The Relative V_{max} values were normalised with respect to the value (%) of that for penicillin.

$$\textbf{Relative } V_{max} = \frac{V_{max} \textbf{ of substrate}}{V_{max} \textbf{ of penicillin}} \times 100$$

3.7.4. Measurement of ß-lactamase inhibition values

The ID_{50} value is defined as the amount of inhibitor required to reduce the enzymatic activity of the ß-lactamase by 50%. The test substrate employed was as stated in the appropriate results section. The initial rate of hydrolysis was determined as described previously, in the absence of inhibitor. This procedure was then repeated in the presence of 10^{-8}M of inhibitor and repeated with increasing ten-fold concentrations of inhibitor until inhibition approached 100%.

The ß-lactamase inhibitors clavulanic acid and BRL 42715 were pre-incubated with the enzyme solution for 5 minutes at 37°C prior to addition of test substrate.

Percentage inhibition was then plotted against $\log_{10}$ concentration of inhibitor. The concentration of the inhibitor which gave 50% inhibition was calculated from the graph.

Some inhibitor studies were performed with a fixed concentration of inhibitor. These results were expressed as % inhibition of rate of reaction compared to a control reaction with no added inhibitor.

3.8. Isolation and purification of DNA

3.8.1. Isolation of plasmid DNA

Three methods were employed for the isolation of plasmid DNA from *A. baumannii* 6B92; these were the methods of, 1)Takahashi and Nagano [220], 2) Hansen and Olsen [221] and 3) Gerner-Smidt [222]

For all methods, cells were grown overnight with shaking in 4.5ml of nutrient broth and harvested by centrifugation (2500 x *g*, 20 minutes, Heraeus Christ, Bactifuge). The methods suggested by the authors were then followed exactly.

3.8.2. Isolation of total genomic DNA

Cells were harvested from 1.2ml of an overnight nutrient broth culture, centrifuged (12500 x *g* for 10 minutes in an MSE Micro Centaur centrifuge), and resuspended in 0.31ml of HTE buffer (50mM Tris Cl, pH 8.0, 20 mM EDTA). Two per cent sarcosyl (0.35ml) in HTE buffer was added and mixed well by inversion. Five µl of RNase was added to remove RNA and the mixture incubated at 37^{o}C for 15 minutes. Thirty five µl of pronase was added and the mixture heated at 50^{o}C until lysis was complete (30-90 minutes). The sample was extracted (to remove lipids and denature proteins) with an equal volume (0.7ml) of phenol chloroform (ratio 1:1) and briefly mixed, centrifuged as before for three minutes to separate the phases. The top layer was carefully removed and transferred to a new microfuge tube. The aqueous DNA layer was extracted a further two times. The sample was then extracted with an equal volume of ether (0.7ml) to remove traces of phenol and chloroform from the aqueous layer. The sample was mixed by inversion and the top layer discarded. Residual ether was evaporated at 60^{o}C for 15 minutes. The DNA was precipitated by the addition of sodium acetate to 0.3M (70µl), mixed well and an equal volume of isopropanol added (0.7ml). After addition of the isopropanol the sample was inverted six times. The sample was then frozen at -70^{o}C for 10 minutes, centrifuged for five minutes and the supernatant discarded. The DNA pellet was washed with ~ 1.0ml of 70% ethanol, vortexed briefly and centrifuged for five minutes to remove residual salts as well as remaining phenol. The pellet was dried under vacuum for 10-20 minutes and resuspended in 50µl of TE buffer (10mM Tris Cl pH 8.0, 0.1 mM EDTA).

The purity of DNA extracted was measured by adding 20µl of DNA preparation to 0.98 ml of TNE buffer (10mM Tris Cl, pH8.0, 10mM NaCl, 0.1mM EDTA). After mixing, the sample was added to a 1 ml quartz cuvette, absorbance was measured at both 260nm and 280nm with a Perkin-Elmer UV/Vis Lambda 2 Spectrophotometer. The ratio of A_{260}/A_{280} was calculated. The purity of the DNA preparation should fall between 1.65 and 1.85. A higher or lower value suggests contamination by phenol or proteins respectively.

The sample was stored at -20°C until required.

3.8.3. Amplification of DNA with the Polymerase Chain Reaction (PCR)

PCR amplification of the TEM structural gene was based on the method described by Du Bois [223].The reaction components are listed in table 20. PCR reaction was performed in a total volume of 100µl prepared in a 0.5ml microfuge tube with a Techne PHC-2 Dri-block Cycler (Cambridge, Cambs.). The mixture was overlaid with mineral oil, the tube was lightly smeared with vacuum grease to ensure efficient heat exchange between the tube contents and the heating block of the thermal cycler. The amplification program was as described in table 21. The sequences of the oligonucleotide primers are shown in Figure 8.

Figure 8.

The sequences of the oligonucleotides, *Bla*3' and *Bla*4'

Bla3':

5'-CTC TCT AGA AAA AGG AAG AGT ATG AGT ATT-3'

Bla4':

5'-CTC GCA TGC GTA AAC TTG GTC TGA CAG TTA-3'

After the amplification procedure was complete the samples were placed on ice to terminate any further reaction. PCR reactions were then ethanol precipitated before verification of DNA amplification by agarose gel electrophoresis and stored at -20°C until required.

Table 20.

PCR reaction components

Component	Quantity	Supplier
10x*Taq* reaction buffer (Mg^{2+} free)	10µl	Promega (Southampton, Hants.)
25mM magnesium chloride	10µl	Promega
4mM dNTP stock solution*	5µl	Böehringer Mannheim, UK.
Bla 3' PCR primer	10pmoles	Oswel laboratory, Dept. of Chemistry, Edinburgh University
Bla 4' PCR primer	10pmoles	Oswel laboratory
Genomic DNA preparation	2µl	
Taq DNA Polymerase	1 unit	Promega
Deionised distilled water (Milli-Q)	to 100µl	

* 50 mM Tris Cl (pH8.0), 76µl Milli-Q-H_20, 1µl 100mM dATP, 1µl 100mM dCTP, 1µl 100mM dTTP, 1 µl 100mM dGTP.

Table 21.

The PCR heating cycle protocol

Segment	Temperature	Time	Repeats	Function
1	96°C	30 sec	x 1	DNA denaturation
	50°C	60 sec		Primer annealing
	72°C	90 sec		Primer extension
2	96°C	15 sec	x 20	DNA denaturation
	50°C	30 sec		Primer annealing
	72°C	90 sec		Primer extension
3	72°C	5 min	-	Final extension

3.8.4. Agarose electrophoresis of DNA

Intact plasmid DNA or PCR-amplified DNA were electrophoresed in 0.7-1.0% W/v agarose gels (Sigma) in TAE buffer (40mM Tris-acetate pH 7.9, 2 mM EDTA). The samples were loaded near the cathode. Electrophoresis was performed on either large (14cm x 25cm x 0.5 cm) or small (Bio-rad Mini Sub Cell) horizontal slab gels submerged in TAE buffer at 70V overnight for the large gels and at 100V for 2 hours for the mini-gels. Each sample was mixed with loading buffer (0.01% W/v bromophenol blue, 50% V/v glycerol) to a ratio of loading buffer to sample of 1: 5, prior to loading onto the gel.

λ phage-DNA digested with *Hin*d III (Sigma) was run alongside samples during electrophoresis.

DNA was visualised after electrophoresis by staining for one hour in a 50mg/l solution of ethidium bromide solution and viewed on a UV transilluminator (UV Products, Cambridge).

Photographs of the stained gel were taken with a Polaroid camera system (setting; F8) for 3 minutes with an orange filter.

The distance travelled by DNA through the gel is inversely proportional to the logarithm of its molecular size. Thus, the unknown sizes were calculated from a graph of $\log_{10}$ molecular size against distance travelled.

CHAPTER 3 RESULTS

1.0. Introduction

Resistance of bacterial pathogens to antimicrobial agents remains a serious problem in spite of the synthesis or discovery of new and novel antimicrobial agents. Traditional methods of monitoring trends in bacterial resistance rely largely on the data of pooled nation-wide statistics detailing the antibiotic susceptibility of pathogens isolated from hospitals over the whole country. These surveys provide general guidelines for treating infections [224] but their value is limited by their inability to detect specific trends within a single hospital.

By analysing susceptibility data over several years, changes can readily be observed in the prevalence of the infecting organisms and the susceptibility to antimicrobials used clinically. Bloodstream monitoring of pathogens has been shown to be an effective means of monitoring the activity of antibiotics and has been used as a primary monitor of pathogenic isolates to detect normal resistance patterns [225].

The Royal Infirmary of Edinburgh is a large teaching hospital and over the past decade, as in other hospitals, there has been a steady increase in the use of more powerful antibiotics for prophylaxis as well as first line drugs in the battle against infection. This is particularly apparent with the use of the third generation cephalosporins and, latterly, imipenem. As a result of the universal increase in use of these agents, there have been many reports of extended-spectrum ß-lactamases produced by Gram negative aerobic bacilli. Recently, there have been reports of these strains isolated in Scotland (Dr C. Thompson, personal communication).

Laboratory policy in the Medical Microbiology Department of Edinburgh University has been to collect and store all significant bacterial isolates from blood cultures from patients in Edinburgh Royal Infirmary. This has given us a unique opportunity to study the incidence of resistance among Gram negative aerobic bacilli over a 12 year period. These strains have been collected from the introduction of extended-spectrum ß-lactams, right through to the present day use of the powerful carbapenems. This information can be of considerable assistance in selecting antimicrobial agents for empiric treatment of sepsis of unclear aetiology.

1.1. The initial aims of the study:

- To study the prevalence of the various species of Gram negative aerobic bacilli responsible for septicaemia over a 12 year study period.

- To determine the *in vitro* susceptibility of these strains to various classes of antibiotics, with particular attention to the ß-lactams.

- To study the role of ß-lactamases in those strains which are resistant to cefuroxime (excluding *Pseudomonas* spp., and *X. maltophilia*, which are normally intrinsically resistant to cefuroxime).

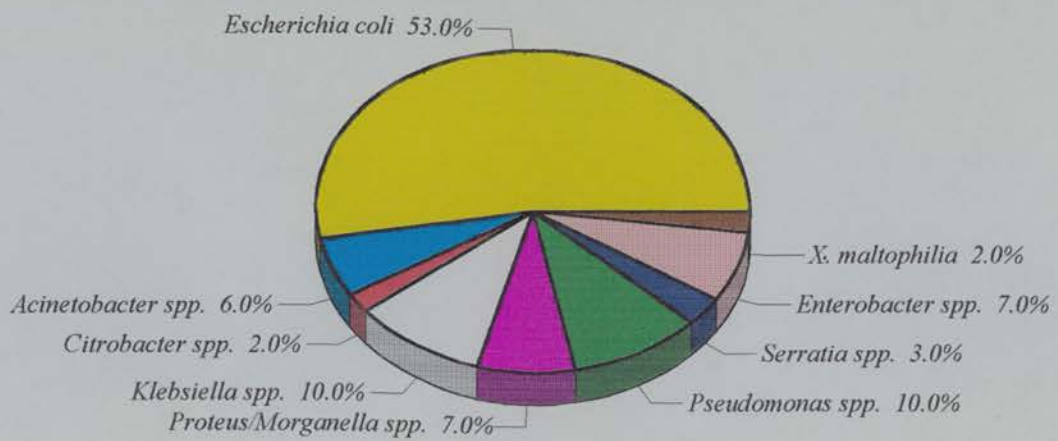
- To determine whether there was any resistance to the newer carbapenem antibiotics; meropenem and imipenem, and to investigate the mechanism of this resistance.

2.0. The antibiotic sensitivity of Gram negative aerobic bacilli isolated from the blood of patients in Edinburgh Royal Infirmary during 1980-1991

Figure 9 shows the percentage of individual species collected during the twelve year study period. As expected *E. coli* was the predominant isolate amongst all Gram negative bacilli examined (53%) with *Klebsiella* spp and *Pseudomonas* spp. the next most prevalent (10%).

Figure 9.

Percentage distribution of Gram negative aerobic bacilli isolated from blood cultures during 1980-91 from Edinburgh Royal Infirmary



Total number of organisms = 1453

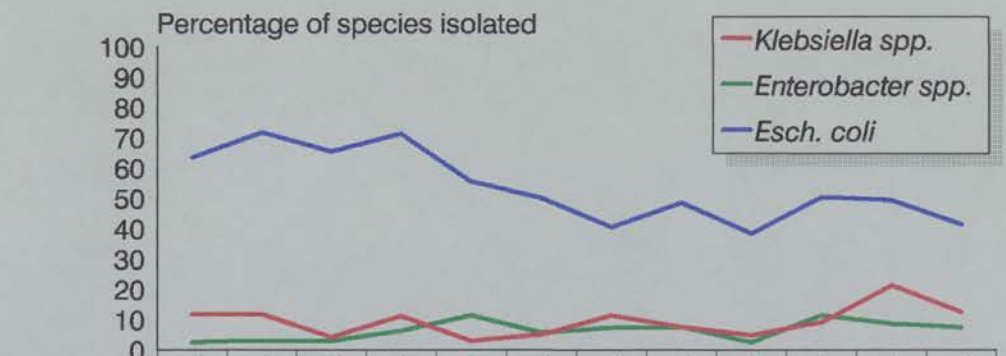
The study strains were further examined to determine whether there had been any change in the incidence of isolation of various species during the study period.

2.1. Prevalence of individual species collected annually, over the twelve year study period.

Figures 10 to 12 show the distribution of each species isolated over the twelve years in both graphical and tabular forms.

Figure 10.

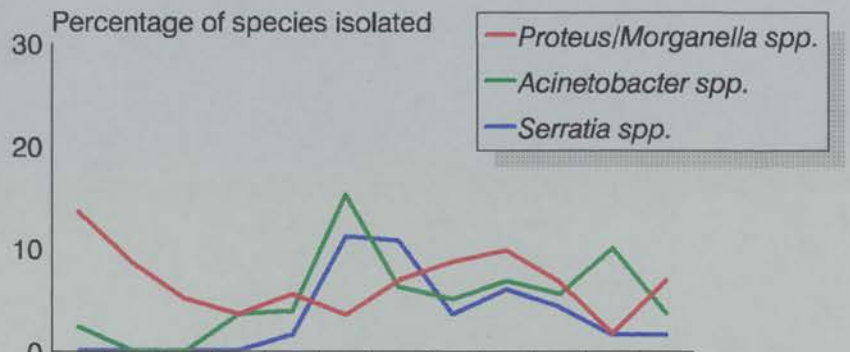
Prevalence of *E. coli*, *Enterobacter* spp., and *Klebsiella* spp. isolated over 12 years from the blood of patients in Edinburgh Royal Infirmary



	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991
Klebsiella spp.	11.3	11.4	3.8	10.8	2.5	4.7	11	7.2	4.5	8.6	20.8	12
Enterobacter spp.	2.2	2.8	2.5	6	11	5.2	6.9	7.2	2.2	11	8.3	6.9
Esch. coli	63	71.4	65	71	55.1	49.7	40	48.2	38	50	49	41

Figure 11.

Prevalence of *Proteus/Morganella* spp., *Acinetobacter* spp., and *Serratia* spp. isolated over 12 years from the blood of patients in Edinburgh Royal Infirmary

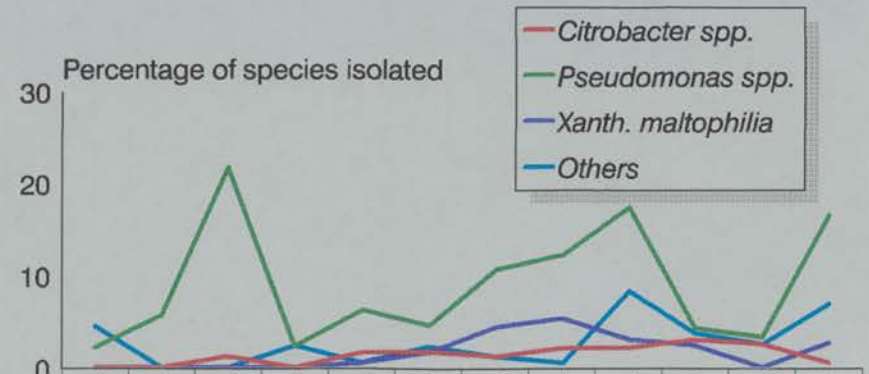


	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991
Proteus/Morganella spp.	13.6	8.6	5.1	3.6	5.5	3.5	6.9	8.6	9.8	6.7	1.7	6.9
Acinetobacter spp.	2.3	0	0	3.6	3.9	15.2	6.2	5	6.8	5.5	10	3.7
Serratia spp.	0	0	0	0	1.6	11.1	10.7	3.6	6	4.3	1.6	1.6

Year isolated

Figure 12.

Prevalence of *Citrobacter* spp., *Pseudomonas* spp., *X. maltophilia* and other miscellaneous Gram negative bacilli isolated over 12 years from the blood of patients in Edinburgh Royal Infirmary



	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991
Citrobacter spp.	0	0	1.2	0	1.6	1.75	1.2	2.1	2.1	3	2.5	0.5
Pseudomonas spp.	2.2	5.7	21.7	2.4	6.2	4.6	10.7	12.2	17.4	4.3	3.3	16.5
Xanth. maltophilia	0	0	0	0	0.5	1.7	4.4	5.3	3	2.4	0	2.7
Others	4.5	0	0	2.4	0.5	2.3	1.2	0.5	8.3	3.7	2.5	6.9

Year isolated

The following conclusions were made:

In parallel with the overall incidence of isolation, *E. coli* remained the most frequently isolated pathogen during the study period, followed by *Klebsiella* spp. There was a general decrease in the percentage of *E. coli* isolated throughout the first six years of the study period, although this percentage levelled out over the final six years of the study, with an average frequency of isolation of 50%. The incidence of *Pseudomonas* spp. isolated, fluctuates greatly over the years, peaking in 1982, with an isolation rate of 21.7%, in contrast with only 3.3% in 1990. Despite the increase in use of later generation cephalosporins over the last decade, there appears to be no obvious trend to the regularity of isolation of any of the other species.

2.2. Minimum inhibitory concentrations of survey strains

MICs were performed on all isolates of Gram negative aerobic bacilli, usually at the time of isolation, to monitor the incidence of resistance to a variety of antimicrobials (the number of antibiotics increasing as newer drugs became available) on either DST or IST agar. It should be noted that for most of the antibiotics used here there is no difference for MIC values between the two media. The exception are MICs performed for *X. maltophilia*. This shall be further discussed in a later section.

Some of the early MIC work was performed by various members of staff from the clinical bacteriology section of the department.

Although recently introduced into the UK, the carbapenems; imipenem and meropenem (not yet licensed for use) and the quinolone ciprofloxacin were added to the survey in 1991. It is expected that the use of these agents will increase in the future. They were therefore included to determine whether there was any underlying resistance to these drugs amongst the survey strains. All strains that were successfully reconstituted from lyophilised cultures were tested against these agents.

The numbers of most of the different species isolated annually were too small to attain statistical significance, and invalidated any attempt to evaluate trends in the incidence of resistance to different antimicrobials over the twelve year period for each species. The incidence of resistance of each species to various antimicrobials was therefore calculated *en bloc*. The minimum inhibitory concentration for 50% and 90% of strains

of strains respectively, against a particular antibiotic (MIC_{50}, MIC_{90} values) and MIC range for all the isolates collectively were determined. Sensitivity breakpoints were as recommended by the British Society for Antimicrobial Chemotherapy Working Party (BSAC) [212].

2.3. Overall resistance of the survey strains to various antimicrobials

Table 22 lists overall MIC data for all species investigated. The highest incidence of resistance to all strains was for ampicillin (63%). Overall resistance to aminoglyosides was surprisingly high (gentamicin 12%, tobramycin 15% and netilmicin 21%) although this may be explained by the number of resistant strains that fell close to the breakpoint. Cumulative MICs for aminoglycosides to all species are shown in figure 13. Ceftazidime was the most effective cephalosporin with 16% resistance, cefotaxime resistance was 21% and as expected, the second generation cephalosporin, cefuroxime was the least effective cephalosporin with 32% resistance. The efficacy of ciprofloxacin was similar to that of imipenem and meropenem.

Figure 13.

Cumulative percentage resistance of Gram negative aerobic bacilli to aminoglycoside antibiotics

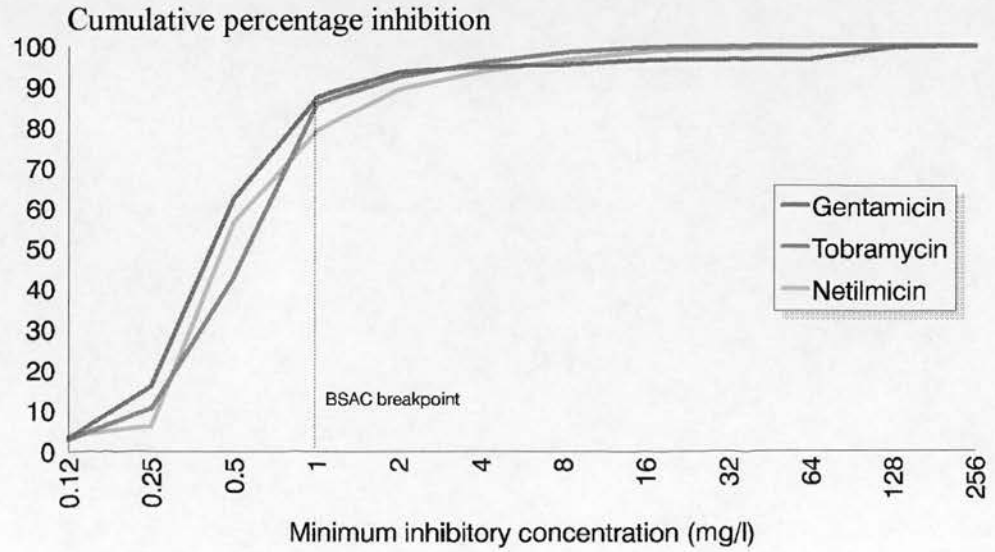


Table 22.

Sensitivity of Gram negative aerobic bacilli isolated during 1980-1991 from blood cultures to 11 antimicrobial agents

Antibiotic	Number tested	(mg/l) MIC_{50}	(mg/l) MIC_{90}	Range	%R	BP
Ampicillin	1431	32	>256	<0.12 - >256	63	8
Gentamicin	1402	0.5	2	<0.12 - >256	12	1
Tobramycin	1027	1	2	<0.12 - >256	15	1
Netilmicin	1016	0.5	2	<0.12 - >256	21	1
Piperacillin	735	2	32	<0.12 - >256	21	16
Cefuroxime	1434	4	256	<0.12 - >256	32	4
Cefotaxime	1441	<0.12	8	<0.12 - >256	21	1
Ceftazidime	737	0.25	4	<0.12 - >256	16	2
Imipenem	1178	0.12	2	<0.008 - >256	4	4
Meropenem	1178	0.016	0.12	<0.018 - >256	2	4
Ciprofloxacin	1176	0.5	0.5	<0.12 - >256	3	1

BP = breakpoint (mg/l)
%R = percentage resistant

2.4. Breakdown of bacterial genera or species and the incidence of resistance within each genus

It is well known that intrinsic resistance within various bacterial species may produce a bias, therefore, for a more informative and complete evaluation of resistance patterns, the incidence of resistance within each genus/species was determined (tables 23-31).

Table 23.

Sensitivity of *Escherichia coli* isolated during 1980-1991 from blood cultures to 11 antimicrobial agents

Antibiotic	Number tested	(mg/l) MIC_{50}	(mg/l) MIC_{90}	Range	%R	BP
Ampicillin	724	8	>256	0.12 - >256	49	8
Gentamicin	718	0.5	1	0.12 - 256	5	1
Tobramycin	535	1	1	0.12 - 16	10	1
Netilmicin	533	0.5	1	0.12 - 16	7	1
Piperacillin	333	2	128	0.12 - >256	25	16
Cefuroxime	723	2	4	0.12 - >256	9	4
Cefotaxime	728	<0.12	0.12	<0.12 - >256	4	1
Ceftazidime	334	<0.12	1	<0.12 - 64	3	2
Imipenem	585	0.03	0.5	<0.008 - 1	0	4
Meropenem	589	0.016	0.016	<0.008 - 0.5	0	4
Ciprofloxacin	585	<0.12	0.06	<0.12 - 0.5	0	1

BP = breakpoint (mg/l)

%R = percentage resistant

Table 24.

Sensitivity of *Klebsiella* spp. isolated during 1980-1991 from blood cultures to 11 antimicrobial agents

Antibiotic	Number tested	(mg/l) MIC_{50}	(mg/l) MIC_{90}	Range	%R	BP
Ampicillin	143	64	>256	2 - >256	83*	8
Gentamicin	137	0.5	1	0.12 >256	5	1
Tobramycin	75	0.5	1	0.12 - 64	5	1
Netilmicin	70	0.5	1	0.12 - 128	4	1
Piperacillin	79	2	8	0.12 - >256	10	16
Cefuroxime	143	2	8	0.12 - >256	9	4
Cefotaxime	143	<0.12	0.12	<0.12 - 2	3	1
Ceftazidime	79	0.12	2	<0.12 - 2	8	2
Imipenem	114	0.25	1	<0.008 - 2	0	4
Meropenem	114	0.016	0.03	<0.008 - 1	0	4
Ciprofloxacin	120	<0.12	0.25	<0.12 - 0.25	0	1

BP = breakpoint (mg/l)

%R = percentage resistant

* See section 2.4.2 p 93

Table 25.

Sensitivity of *Proteus/Morganella* spp isolated during 1980-1991 from blood cultures to 11 antimicrobial agents

Antibiotic	Number tested	(mg/l) MIC_{50}	(mg/l) MIC_{90}	Range	%R	BP
Ampicillin	89	4	>256	0.25 - >256	42	8
Gentamicin	86	0.5	2	0.25 - 2	10	1
Tobramycin	66	0.5	2	0.12 - 4	9	1
Netilmicin	67	0.5	2	<0.12 - 64	15	1
Piperacillin	50	0.5	16	<0.12 - 64	6	16
Cefuroxime	91	2	32	<0.12 - 128	19	4
Cefotaxime	91	<0.12	0.12	<0.12 - 8	4	1
Ceftazidime	50	<0.12	1	<0.12 - 4	4	2
Imipenem	72	1	>4	0.03 - >4	10	4
Meropenem	72	0.06	0.25	<0.008 ->4	2.8	4
Ciprofloxacin	72	<0.12	0.25	<0.12 - 2	4	1

BP = breakpoint (mg/l)

%R = percentage resistant

Table 26.

Sensitivity of *Enterobacter* spp. isolated during 1980-1991 from blood cultures to 11 antimicrobial agents

Antibiotic	Number tested	(mg/l) MIC_{50}	(mg/l) MIC_{90}	Range	%R	BP
Ampicillin	97	64	>256	0.25 - >256	87	8
Gentamicin	95	0.5	64	<0.12 - 128	14	1
Tobramycin	63	1	8	<0.12 - 16	25	1
Netilmicin	61	0.5	16	1 - >256	21	1
Piperacillin	54	2	>256	1 - >256	15	16
Cefuroxime	97	8	>256	0.25 - >256	70	4
Cefotaxime	98	0.25	8	<0.12 - 128	24	1
Ceftazidime	54	0.5	16	<0.12 - 128	22	2
Imipenem	83	0.5	1	0.016 - 2	0	4
Meropenem	83	0.03	0.06	<0.008 - 0.25	0	4
Ciprofloxacin	83	<0.12	0.25	<0.12 - 2	4	1

BP = breakpoint (mg/l)

%R = percentage resistant

Table 27.

Sensitivity of *Acinetobacter* spp. isolated during 1980-1991 from blood cultures to 11 antimicrobial agents

Antibiotic	Number tested	(mg/l) MIC_{50}	(mg/l) MIC_{90}	Range	%R	BP
Ampicillin	86	32	128	<0.12 - >256	74	8
Gentamicin	84	1	2	<0.12 - 128	18	1
Tobramycin	67	1	8	<0.12 - 32	30	1
Netilmicin	66	2	32	<0.12 - 256	55	1
Piperacillin	42	16	64	0.5 - >256	48	16
Cefuroxime	87	16	64	<0.12 - 128	77	4
Cefotaxime	88	8	16	<0.12 - 128	84	1
Ceftazidime	42	4	32	<0.12 - 128	71	2
Imipenem	73	0.06	0.25	0.008 - >4	0.7	4
Meropenem	75	0.25	0.5	<0.008 - >4	0.7	4
Ciprofloxacin	74	0.25	1	<0.12 - 4	5	1

BP = breakpoint (mg/l)

%R = percentage resistant

Table 28.

Sensitivity of *Citrobacter* spp. isolated during 1980-1991 from blood cultures to 11 antimicrobial agents

Antibiotic	Number tested	(mg/l) MIC_{50}	(mg/l) MIC90	Range	%R	BP
Ampicillin	25	256	>256	1 - >256	76	8
Gentamicin	24	0.5	64	0.25 - 128	17	1
Tobramycin	20	1	8	0.25 - 8	30	1
Netilmicin	20	0.5	8	0.12 - 16	25	1
Piperacillin	17	4	64	1 - >256	18	16
Cefuroxime	25	8	64	0.25 - >256	56	4
Cefotaxime	25	0.25	2	<0.12 - 32	28	1
Ceftazidime	17	2	16	<0.12 - 64	29	2
Imipenem	20	0.25	1	0.016 - 2	0	4
Meropenem	20	0.03	0.25	<0.008 - 2	0	4
Ciprofloxacin	20	0.06	0.5	<0.12 - 1	0	1

BP = breakpoint (mg/l)

%R = percentage resistant

Table 29.

Sensitivity of *Serratia* spp. isolated during 1980-1991 from blood cultures to 11 antimicrobial agents

Antibiotic	Number tested	(mg/l) MIC_{50}	(mg/l) MIC_{90}	Range	%R	BP
Ampicillin	64	16	>256	0.25 - >256	69	8
Gentamicin	59	0.5	1	0.25 - 4	3.4	1
Tobramycin	57	1	2	<0.12 - 4	19	1
Netilmicin	54	0.5	2	<0.12 - 4	30	1
Piperacillin	25	2	4	<0.12 - 8	0	16
Cefuroxime	64	32	256	1 - >256	89	4
Cefotaxime	64	0.12	2	<0.12 - 8	9	1
Ceftazidime	25	0.25	2	<0.12 - 4	12	2
Imipenem	44	0.25	1	0.016 - 2	0	4
Meropenem	44	0.03	0.06	<0.008 - 2	0	4
Ciprofloxacin	44	0.06	0.5	<0.12 - 1	0	1

BP = breakpoint (mg/l)

%R = percentage resistant

Table 30.

Sensitivity of *Pseudomonas* spp. isolated during 1980-1991 from blood cultures to 11 antimicrobial agents

Antibiotic	Number tested	(mg/l) MIC_{50}	(mg/l) MIC_{90}	Range	%R	BP
Ampicillin	129	>256	>256	<0.12 - >256	95	8
Gentamicin	125	1	4	0.5 - >256	44	1
Tobramycin	92	0.5	1	<0.12 - 256	8	1
Netilmicin	93	2	8	0.25 - 32	66	1
Piperacillin	81	4	64	<0.12 - 64	15	16
Cefuroxime	129	256	>256	<0.12 - >256	88	4
Cefotaxime	129	4	8	<0.12 - >256	88	1
Ceftazidime	82	2	32	<0.12 - 128	42	2
Imipenem	114	2	>4	<0.008 - >4	10	4
Meropenem	114	0.25	1	<0.008 - >4	3.5	4
Ciprofloxacin	113	0.25	1	<0.12 - 8	6	1

BP = breakpoint (mg/l)

%R = percentage resistant

Table 31.

Sensitivity of *Xanthomonas maltophilia* isolated during 1980-1991 from blood cultures to 11 antimicrobial agents

Antibiotic	Number tested	(mg/l) MIC_{50}	MIC_{90}	Range	%R	BP
Ampicillin	31	64	>256	1 - >256	97	8
Gentamicin	30	16	64	0.5 - 128	83	1
Tobramycin	25	8	32	0.25 - 64	72	1
Netilmicin	26	8	128	<0.12 - 256	81	1
Piperacillin	23	16	64	4 - 64	26	16
Cefuroxime	31	>256	>256	>256	100	4
Cefotaxime	31	16	64	1 - 128	87	1
Ceftazidime	23	4	16	0.5 - 16	50	2
Imipenem	24	>4		>4	100	4
Meropenem	24	>4		>4	100	4
Ciprofloxacin	24	8	64	0.5 - 64	87.5	1

BP = breakpoint (mg/l)

%R = percentage resistant

In brief, the results of individual species are as follows:

2.4.1. *Escherichia coli*

E. coli comprised the largest proportion of species tested. The penicillins, ampicillin and piperacillin (49% and 25% resistance respectively) proved to be the least effective antimicrobial against this pathogen. In general, the aminoglycosides exhibited a slightly higher level of resistance than the second and third generation cephalosporins tested. The newer antibiotics, meropenem and imipenem (carbapenems) and the quinolone, ciprofloxacin were extremely active against this species, with no resistance to these drugs from any of the strains of *E.coli* tested.

2.4.2. *Klebsiella* spp.

Apart from ampicillin (83% resistance, although it is generally accepted that all *Klebsiella* spp. are intrinsically resistant to ampicillin), the incidence of resistance to all other antimicrobials was similar to that of *E. coli*, although resistance to piperacillin was 15% less than that to *E. coli*. Again, there was no resistance to the carbapenems or to ciprofloxacin.

2.4.3. *Proteus/Morganella spp.*

Proteus/Morganella spp. were more resistant to cefuroxime (19% resistance) than either *E. coli* or *Klebsiella* spp. Three strains were resistant to ciprofloxacin, although the MIC values for all three isolates were only one doubling dilution higher than the breakpoint value. The *Proteeae* were the only members of the Enterobacteriaceae to demonstrate any resistance to the carbapenems, with 10% resistance to imipenem and 2.8% resistance to meropenem respectively.

2.4.4. Species that produce a Class I cephalosporinase

In general, all these species were much more resistant to almost all classes of antimicrobials tested, than were the other genera, particularly to the cephalosporins,

aminoglycosides and penicillins. There was virtually no resistance to the two carbapenems. One strain of *Acinetobacter baumannii* exhibited resistance to these drugs. Ciprofloxacin was again a very useful agent, highly effective against all strains examined.

2.4.5. *Pseudomonas* spp.

Pseudomonas spp. as expected, were resistant to most classes of antimicrobials. Tobramycin was the most effective aminoglycoside of the three examined. The carbapenems and ciprofloxacin remained active for most strains.

2.4.6. *Xanthomonas maltophilia*

X. maltophilia proved to be resistant to most of the antimicrobials tested. Piperacillin proved to be the most useful with only 26% of strains resistant. It has been recently reported that the type of media used, can affect the susceptibility of *X. maltophilia* to various compounds [226-228]. These results may not truly reflect the level of resistance. The susceptibility of this species shall be examined in more depth in a later section (5.2.).

In conclusion:

The survey demonstrated that there was a higher than expected level of resistance to almost all classes of antimicrobials, including later generation cephalosporins and aminoglycosides. Only the carbapenems and ciprofloxacin showed excellent activity against most isolates.

One of the original aims of this thesis was to establish the resistance mechanism of these strains to later generation cephalosporins and 245 cefuroxime resistant strains were studied in further detail in order to investigate the resistance mechanisms involved.

3.0. Cefuroxime-resistant strains

Strains from the main survey, that were resistant to cefuroxime (>4 mg/l, as recommended by BSAC guidelines) were selected to examine their ß-lactamase complement. All strains of *Pseudomonas* spp. and *X. maltophilia* were excluded. The distribution of species is shown in table 32. Their sensitivity values and percentage resistance to a range of antimicrobials is shown in table 33.

Table 32.

Distribution of species of blood culture isolates resistant to cefuroxime (No=245)

Species/Genus	Number detected
Enterobacter spp.	61
Acinetobacter spp.	59
Serratia spp.	37
E. coli	44
Klebsiella spp.	11
Citrobacter spp.	12
Proteus/Morganella spp.	15
Others	6

The cefuroxime-resistant population demonstrated the presence of a large proportion of organisms such as *Enterobacter* spp. and *Serratia* spp. that are known to produce an inducible Class I cephalosporinase. There are also a large number of *Acinetobacter* spp. present (59), although it is of some debate whether these organisms produce a typical inducible cephalosporinase. There was a surprising number of *E. coli* isolates resistant to cefuroxime (44). On closer scrutiny of the MIC data, it was seen that 24 of these strains fell only one dilution above the breakpoint value (data not shown).

Table 33.

Sensitivity values for the 245 cefuroxime resistant strains to other antimicrobials

Antibiotic	Number tested	% Resistant	Break point (mg/l)
Ampicillin	245	93	8
Gentamicin	242	15	1
Tobramycin	152	29	1
Netilmicin	151	39	1
Piperacillin	150	27	16
Cefuroxime	245	100	4
Cefotaxime	245	46	1
Ceftazidime	150	36	2
Imipenem	225	0.8	4
Meropenem	225	0.4	4
Ciprofloxacin	226	2.2	1

As expected almost all the cefuroxime-resistant strains were also resistant to ampicillin. Gentamicin was the most effective of the aminoglycosides, with an overall resistance of 15%. Resistance to ciprofloxacin was 2.2%. The carbapenems demonstrated the highest level of activity against all species, with <1.0% resistance for both drugs.

These results demonstrate that the cefuroxime-resistant population displays cross resistance to other classes of antimicrobials. Only one strain was resistant to both carbapenems. This was a strain of *A. baumannii* 6B92.

3.1. Analytical isoelectric focusing of the ß-lactamases produced by the cefuroxime-resistant population

The ß-lactamase complement of all the cefuroxime-resistant strains was determined by analytical isoelectric focusing of small-scale crude ß-lactamase extracts. The plasmid mediated ß-lactamases, TEM-1 and SHV-1 were used as standard ß-lactamases. The gels were also quantified with the use of standard isoelectric point marker enzymes. This allowed for the presumptive identification of the ß-lactamases present as to whether they were of plasmid or chromosomal origin. A representative gel is shown (see figure 14). The distribution of the presumptive ß-lactamases present in the 245 cefuroxime-resistant isolates is shown in table 34. No attempt was made to distinguish between chromosomal ß-lactamase types e.g. *E. cloacae* P99 ß-lactamase, and these ß-lactamases are simply referred to as chromosomal ß-lactamases.

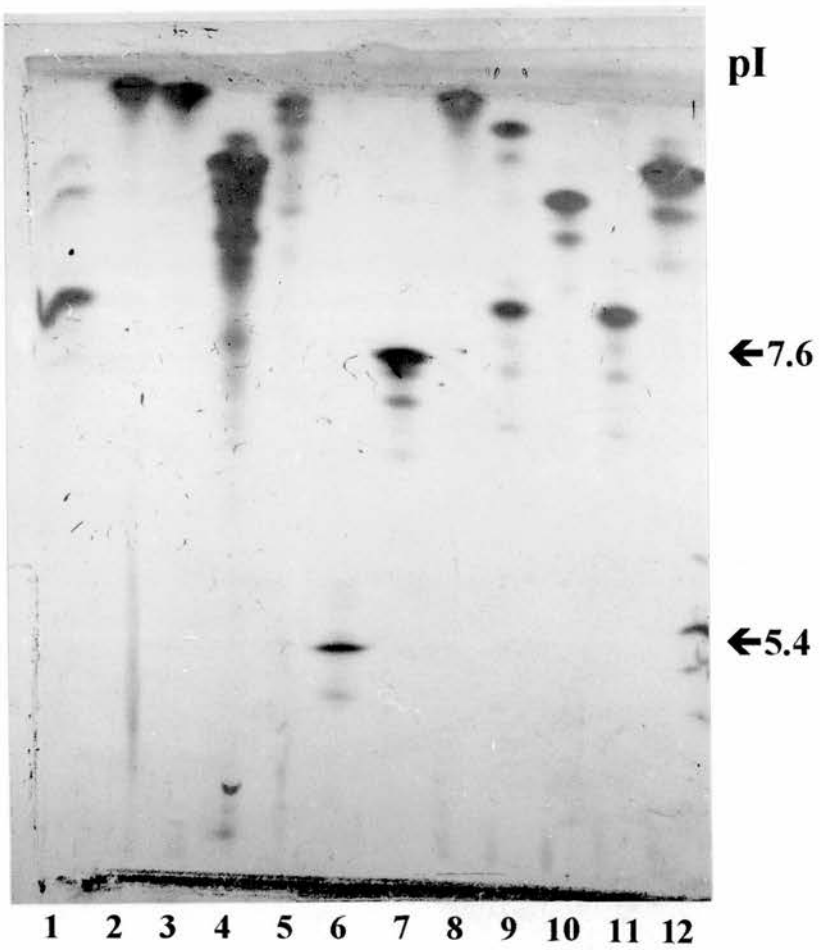
Table 34.

Distribution of ß-lactamases present in the 245 cefuroxime isolates examined

Bacterial species	Chromosomal ß-lactamase alone	Chromosomal and plasmid ß-lactamases	Plasmid ß-lactamase alone	None	Uncertain identification
Enterobacter spp.	49	10 (TEM-1)	1 (SHV-1)	1	
Acinetobacter spp.	56	1 (TEM-1)		1	1 (pI 6.65)
Serratia spp.	36	1 (TEM-1)			
Escherichia coli	22	14 (TEM-1)	4 (TEM-1) 3 (SHV-1)	1	
Klebsiella. spp.	8	1 (SHV-1)	2 (SHV-1)		
Citrobacter spp.	10	2 (TEM-1)			
Proteus/ Providencia/ Morganella spp.	13			2	
Others	4			2	
Total	**198**	**28 (TEM-1) 1 (SHV-1)**	**4 (TEM-1) 6 (SHV-1)**	**7**	**1**

Figure 14.

Analytical isoelectric focusing of ß-lactamases from cefuroxime-resistant strains



IEF gel contained ampholines pH range 3.5-10.6.

Lane 1:	*Achromobacter xylosoxidans*	9B49
Lane 2:	*Acinetobacter anitratus*	9B138
Lane 3:	*Acinetobacter anitratus*	9B208
Lane 4:	*Citrobacter freundii*	9B219
Lane 5:	*Acinetobacter anitratus*	9B322
Lane 6:	TEM-1 ß-lactamase (pI 5.4)	
Lane 7:	SHV-1 ß-lactamase (pI 7.6)	
Lane 8:	*Acinetobacter anitratus*	9B330
Lane 9:	*Serratia marcescens*	9B337
Lane 10:	*Serattia marcescens*	9B364
Lane 11:	*Enterobacter spp.*	10B38
Lane 12:	*Serratia spp.*	10B53

In summary, of 245 cefuroxime resistant strains examined; 198 produced a chromosomal enzyme alone, 28 produced a chromosomal enzyme plus the TEM-1 ß-lactamase, 1 produced a chromosomal ß-lactamase plus the SHV-1 ß-lactamase, 6 produced the SHV-1 ß-lactamase alone. Four produced the TEM-1 ß-lactamase alone. Seven strains failed to produce a detectable ß-lactamase. One strain of *A. baumannii* 6B92 produced a chromosomal enzyme and an unknown ß-lactamase, that focused with a pI of 6.65.

These findings would suggest that the cefuroxime resistance in most of these strains would result from the production of a 'typical' chromosomal ß-lactamase. No strain appeared to produce any of the extended-spectrum plasmid-mediated enzymes, derived from the common plasmid-mediated ß-lactamases, TEM-1 and SHV-1. To attempt to confirm this statement, conjugation experiments were performed on the 245 cefuroxime strains. It should be noted, however, that other mechanisms of resistance such as permeability and change in affinity for PBPs should not be discounted.

3.2. Conjugation experiments

Conjugation experiments were carried out with *E. coli* J62-2. *E. coli* J62-2 transconjugants were selected on suitably supplemented DM agar containing ampicillin (10mg/l) and rifampicin (25mg/l). Resistance patterns of the transconjugants were determined by Stokes' disc sensitivity testing on IST agar.

The results of the conjugation experiments are shown in table 35. Twenty transconjugants were obtained, and of these, 18 produced the TEM-1 ß-lactamase while 2 produced SHV-1. It was concluded that the other presumed plasmid-mediated ß-lactamases in the survey strains that were not transferred, were either carried on non-transmissible plasmids, or that they had integrated into the host cell chromosome.

There was no evidence for production of plasmid-mediated, extended-spectrum ß-lactamases in these strains. Only the well known TEM-1 and SHV-1 ß-lactamases were encoded by the transconjugants. These results further substantiated the statement that production of chromosomal cephalosporinases are largely responsible for cefuroxime resistance in the survey strains.

Table 35.

Results of conjugation experiments on the cefuroxime resistant isolates

Species	Blood culture number	ß-lactamase transferred	Resistance markers of transconjugants
E. coli	1B4	TEM-1	Ap, Tp
E. coli	1B7	TEM-1	Ap, Tp
E. coli	1B51	TEM-1	Ap, Tp
E. coli	1B39	TEM-1	Ap, Tp
E. coli	1B104	TEM-1	Ap, Tp, Te
K. pneumoniae	2B64	SHV-1	Ap, Tp
E. coli	3B28	TEM-1	Ap, Tp
E. coli	3B91	TEM-1	Ap
E. coli	4B115	SHV-1	Ap
E. coli	5B90	TEM-1	Ap, Sm, Te
E. coli	5B101	TEM-1	Ap, Te
E. cloacae	6B242	TEM-1	Ap
E. cloacae	8B491	TEM-1	Ap
E. cloacae	9B230	TEM-1	Ap
E. coli	11B46	TEM-1	Ap, Te
E. coli	11B75	TEM-1	Ap, Sm, Te
E. coli	11B218	TEM-1	Ap, Te
E. coli	11B351	TEM-1	Ap, Sm
E.coli	12B453	TEM-1	Ap
E. coli	12B458	TEM-1	Ap

Abbreviations: Ap, ampicillin; Sm, streptomycin; Te, tetracycline, Tp, trimethoprim.

4.0. Carbapenem resistance among the cefuroxime-resistant survey strains

Another aim of this thesis was to examine any strain in the survey of cefuroxime resistant strains that exhibited resistance to carbapenems. As previously mentioned, one strain, *Acinetobacter baumannii* 6B92, was resistant to both carbapenems (MIC>4 mg/l). This strain was isolated in 1985 from a 55 year old male from a surgical ward in Edinburgh Royal Infirmary. Resistance to carbapenems in clinical isolates of *Acinetobacter* spp. is very rare, and is usually the result of a presumed permeability mutation, or altered PBPs, as a consequence of the heavy use of imipenem. In this particular instance, imipenem was not available for clinical use at the time.

The resistance mechanism to carbapenems in this strain was studied further.

4.1. *Acinetobacter baumannii* 6B92: Investigation of the resistance mechanism to carbapenems

The mechanism of this resistance was investigated to determine whether it might be ß-lactamase-mediated. The biochemical characterisation of the ß-lactamases studied, essentially follows the recommendations for the evaluation of novel ß-lactamases as proposed by Bush and Sykes [171].

4.2. Susceptibility testing

Stokes' disc sensitivity and MIC determinations are shown in Table 36. *A. baumannii* 6B92 was resistant to all penicillins tested and resistant or of intermediate sensitivity to all classes of cephalosporins. It was resistant to both the carbapenems, with MIC values of 16.0 and 32.0 mg/l for imipenem and meropenem respectively. Only ciprofloxacin (0.25 mg/l) and gentamicin (0.5 mg/l) were effective against this strain.

Table 36.

Disc susceptibility results and MICs of various antibiotics to *A. baumannii* 6B92

Antibiotic	Disc content (µg)	*Acinetobacter baumannii* 6B92 Disc	MIC (mg/l)
Ampicillin	10	Resistant	>256
Piperacillin	75	Resistant	-
Azlocillin	75	Resistant	128
Amp/Clavulanate*	30	Resistant	>128
Cephaloridine	30	Resistant	-
Cephalexin	30	Resistant	-
Cefuroxime	30	Resistant	32
Cefotaxime	10	Intermediate	16
Ceftazidime	10	Intermediate	4
Cefoxitin	10	Resistant	-
Trimethoprim	2.5	Resistant	-
Gentamicin	10	Sensitive	0.5
Imipenem	10	Resistant	16
Meropenem	-	-	32
Aztreonam	30	Resistant	-
Ciprofloxacin	1	Sensitive	0.25

* 2:1 ratio. The results are expressed in terms of ampicillin,

-; not tested.

4.3. Isoelectric focusing

A large-scale ß-lactamase preparation was prepared from 1L of nutrient broth. Isoelectric focusing of the preparation, employing a broad range of ampholines (pH 3.5-10.6), revealed the presence of two ß-lactamases. One enzyme produced several satellite bands with a main band focusing at pI 6.65. The other enzyme focused poorly and spread up the gel towards the cathode, with a pI of > 9.0 (figure 15).

It is known that all strains of *Acinetobacter* spp. produce a chromosomal cephalosporinase of high pI, whereas the other ß-lactamase present of pI 6.65 differs from any other ß-lactamase found in *Acinetobacter* spp.

This was a significant finding, and the enzymes were partially purified in order to study their properties in more detail.

4.4. Molecular mass determination (M_r)

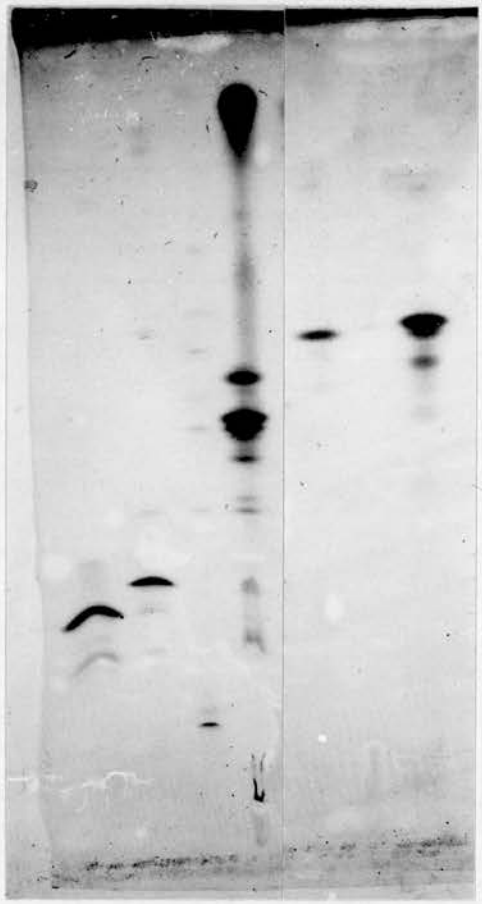
Both these enzymes were partially purified and their M_r determined by gel filtration using a pre-calibrated Sephadex G-75 gel filtration column as described in Materials and Methods. The samples were eluted with 50mM sodium phosphate buffer (pH 7.0). The elution of the standard proteins and ß-lactamase activity from the G-75 column are shown in figure 16.

A standard curve of $\log_{10}$ molecular mass versus fraction number was drawn (data not shown), and the M_r of the ß-lactamases determined.

The enzyme of pI>9.0 had an M_r of 58000 whereas the enzyme of pI 6.65 had an M_r of 23000.

Figure 15.

Isoelectric focusing of *Acinetobacter baumannii* 6B92 ß-lactamases

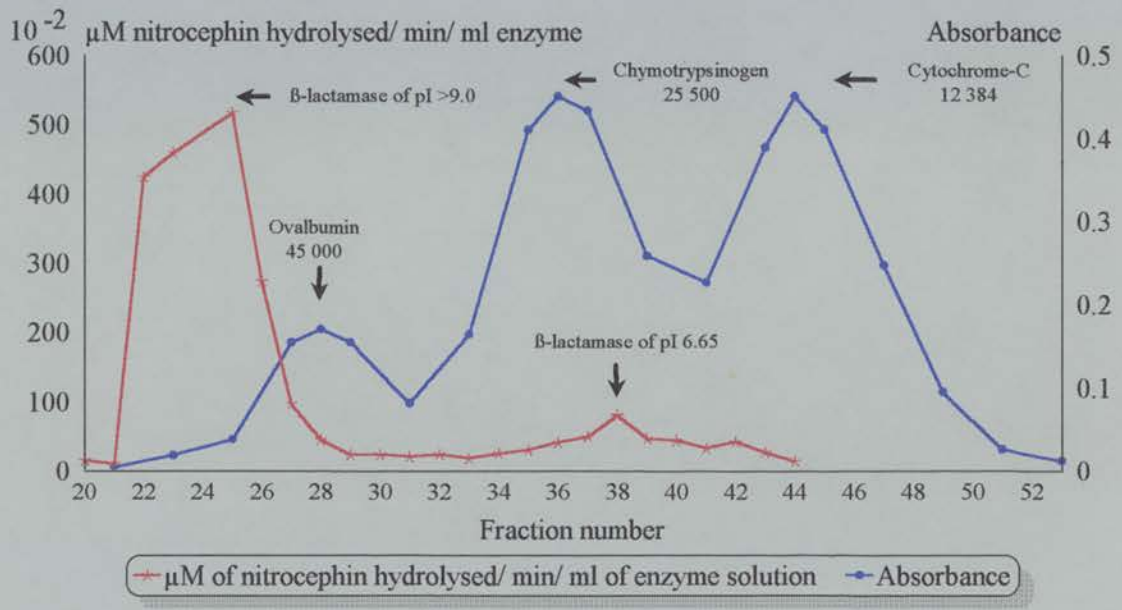


IEF gel contained ampholines pH range 3.5-10.6

Lane 1:	TEM-1 ß-lactamase	(pI 5.4)
Lane 2:	TLE-1 ß-lactamase	(pI 5.55)
Lane 3:	wide range pI markers	4.7-10.6
Lane 4:	*Acinetobacter baumannii* 6B92	
Lane 5:	OXA-1 ß-lactamase	(pI 7.4)
Lane 6:	OXA-4 ß-lactamase	(pI 7.5)
Lane 7:	SHV-1 ß-lactamase	(pI 7.6)

Figure 16.

Relative rate of hydrolysis of nitrocephin by ß-lactamases from *Acinetobacter baumannii* 6B92 eluted from G-75 Sephadex column



The isoelectric points and M_r determinations of the two ß-lactamases suggested that the enzyme of high pI was indeed a 'typical' chromosomal cephalosporinase of the type described by Hood and Amyes [229]. They are enzymes of high molecular mass, and have been shown to focus poorly in conventional systems [229]. These chromosomal ß-lactamases have been shown to have no activity against carbapenems [188]. Attention was therefore centred on the enzyme that focused sharply at pI 6.65, and enzyme kinetic experiments were performed to determine whether this ß-lactamase was capable of hydrolysing carbapenems.

4.5. Hydrolysis of ß-lactam antibiotics by the ß-lactamase of pI 6.65 from *Acinetobacter baumannii* 6B92

The enzyme preparation was concentrated before use in a centriprep® 10 concentrator (Amicon, Danvers, USA). The Michaelis constant (K_m) and the maximum rate of hydrolysis (V_{max}) of the pI 6.65 enzyme were determined by assaying the enzyme in decreasing substrate concentrations. The results are shown in table 37. The values are expressed as a percentage of the value for penicillin.

Table 37.

Hydrolysis of ß-lactam antibiotics by the ß-lactamase of pI 6.65 from *Acinetobacter baumannii* 6B92

Substrate	V_{max}[b]	Relative V_{max}[a]	K_m[c]
Penicillin	0.128	100	0.048
Ampicillin	0.196	153	0.139
Cephaloridine	0.07	35.7	0.083

[a] Relative to penicillin 100%
[b] µmol of substrate hydrolysed/min /ml of enzyme solution
[c] mmol of substrate

Penicillin and ampicillin were hydrolysed weakly. The first generation cephalosporin, cephaloridine was hydrolysed at about a third the rate of penicillin. Azlocillin was also hydrolysed, but so slowly that it was impossible to calculate V_{max} and K_m values. Hydrolysis of imipenem, meropenem, cefuroxime, cefotaxime and ceftazidime could not be demonstrated by spectrophotometric methods even after prolonged assay times.

These results do not show any evidence for carbapenemase activity; however, the substrate profile of the ß-lactamase presents that of a penicillinase rather than a cephalosporinase.

4.6. Microbiological assay

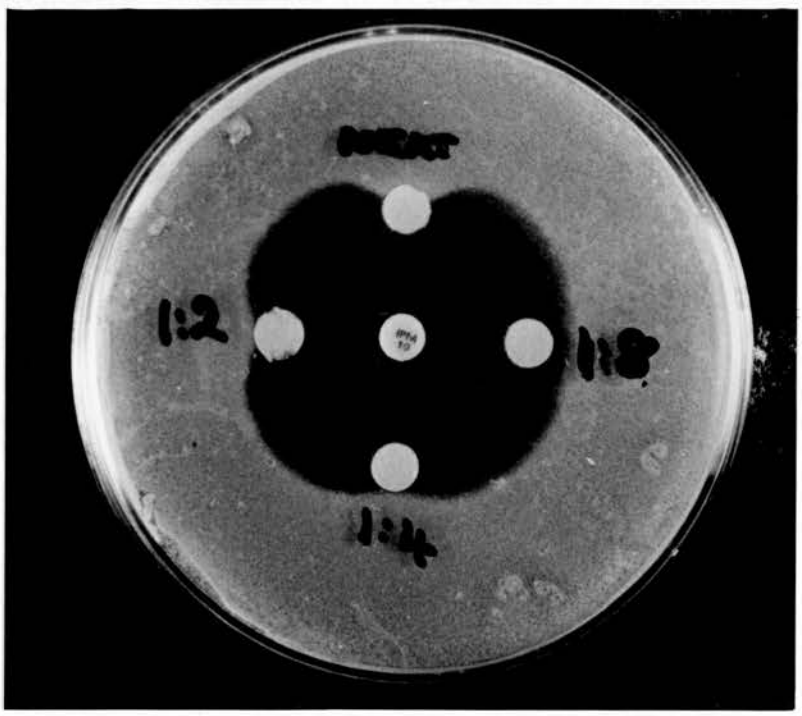
Although convenient, it is known that spectrophotometry is not suitable for assay of all ß-lactamases. It was therefore decided to employ a microbiological assay. Although time consuming, microbiological assays have been shown to be extremely sensitive [230]. The protocol was essentially that described by Masuda *et al.* [230]. The indicator organism employed was *S. aureus* NCTC 6571. Filter paper pads containing 15µl of enzyme preparation neat or diluted 1:2, 1:4 or 1:8 in 50mM phosphate buffer (pH 7.0) were placed at the periphery at the expected zone of inhibition to the antibiotic discs.

4.6.1. Result

Any inactivation of substrate by the enzyme preparation was indicated by growth of the indicator strain within the pre-determined zone of inhibition of the antibiotic. The more dilute the enzyme preparation, the weaker the inhibition. This technique clearly confirmed inactivation of imipenem (figure 17) and azlocillin (figure 18) by the ß-lactamase of pI 6.65. The zones of inhibition to the indicator strain by cefuroxime, ceftazidime and cefotaxime were unaffected, confirming that these substrates were not inactivated by the enzyme (figure 18).

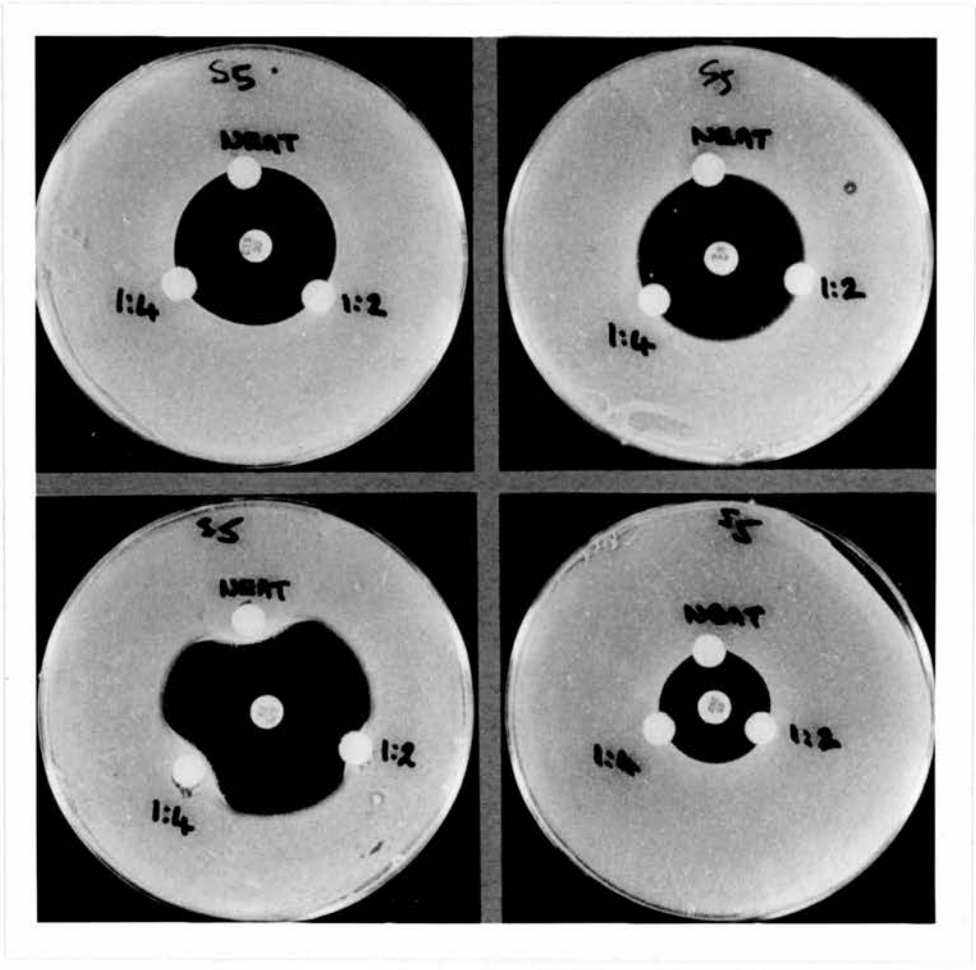
This enzyme was thus designated **ARI-1** (*Acinetobacter* resistant to imipenem).

Figure 17.



Microbiological assay plate showing inactivation of imipenem by *Acinetobacter baumannii* 6B92 ARI-1 ß-lactamase

Figure 18.



Microbiological assay plate showing action of *Acinetobacter baumannii* ARI-1 ß-lactamase on various ß-lactams

1: Cefotaxime

2: Cefuroxime

3: Azlocillin

4: Ceftazidime

4.7. Inhibitor studies

The effects of inhibitors, expressed as the concentration required for 50% inhibition of enzyme activity (ID_{50}) are shown in table 38. TEM-1 was included as a reference ß-lactamase.

The ARI-1 enzyme required much higher amounts of clavulanate for inhibition than TEM-1. P-chloromercuribenzoic acid (p-CMB) and EDTA did not inhibit the ARI-1 enzyme. Non-inhibition by EDTA would imply that metal ions play no part in the catalytic activity of the enzyme, suggesting that ARI-1 functions by virtue of a serine residue at the active site. ARI-1 was inhibited by BRL 42715, again suggesting the presence of a catalytically active serine residue at the active site. TEM-1 was much less susceptible to inhibition with imipenem and meropenem than the ARI-1 ß-lactamase. This indicates that the ARI-1 ß-lactamase binds carbapenems.

Table 38.

ID_{50} values of various inhibitors using nitrocephin (50 mg/l) as reporter substrate

Inhibitor	ID_{50} (µM)	
	ARI-1	TEM-1
p-CMB	>100	>100
Clavulanate	100	0.5
BRL 42715	0.001	Not tested
EDTA	>10000	>10000
Meropenem	5.5	>100
Imipenem	0.0875	>100

4.8. Genetic studies

Isolation of any plasmid DNA was unsuccessful despite the utilisation of three differing plasmid DNA isolation techniques [220-222]. All these methods are essentially based on alkaline denaturation of chromosomal DNA but with differing procedures for extraction and purification of plasmid DNA. One method has been shown to be especially suitable for isolation of plasmid DNA from *Acinetobacter* spp. [222].

Several of the gels confirmed the presence of a small plasmid (6.5kb), however the physical loss of this plasmid could not be demonstrated in the cured strain. The small size of this plasmid and its continued presence in the cured strain would make it unlikely that it harboured the gene responsible for carbapenem resistance. The failure to demonstrate physically the presence of a plasmid would therefore suggest the gene is located on a large plasmid, which has broken up during the extraction procedure.

Transfer of resistance was attempted by conjugation to *E. coli* J62-2, *Ps. aeruginosa* PAO8 and a wild strain of *A. baumannii*. No transfer was obtained, suggesting the possibility of a small plasmid unable to code for its own transfer. Mobilisation of resistance was then attempted with the *inc*P plasmid RP4. No transfer of resistance was obtained, despite varying the incubation temperature (30^{o}C and 37^{o}C) and times of incubation of the donor and recipient.

No transfer of plasmid DNA was detected by transformation to *E. coli* C600, it is known that small pieces of DNA are transferred more efficiently by transformation than large molecules, which would suggest again the possibility of a large plasmid.

It was concluded from the results of the genetic experiments that the gene that encodes for ARI-1 was either located on the host cell chromosome or more probably on a large plasmid, which has not been visualised or transferred.

4.9. Plasmid curing experiments

In a final attempt to assert the extrachromosomal location of the genetic determinant responsible for carbapenem resistance it was decided to employ plasmid 'curing' experiments. Initial attempts involved the use of acridine orange as the curing agent. Acridine orange preferentially inhibits plasmid replication [231]. The method used was that outlined by Caro *et al.*[231].

4.9.1. Plasmid curing with acridine orange

Ten ml doubling dilutions of acridine orange (0.12 mg/l - 1024 mg/l) were prepared in nutrient broth, and 20µl of an overnight broth culture of *A. baumannii* 6B92, grown at 37°C was added. After overnight incubation at 37°C it was observed that *A. baumannii* 6B92 was resistant to high concentrations of acridine orange (>1024 mg/l), and this dye was therefore unsuitable for plasmid curing for this particular isolate. Another agent was required.

4.9.2. Plasmid curing with ethidium bromide

Ethidium bromide, a trypanocidal drug affecting nucleic acid was chosen. This agent has been shown to eliminate some antibiotic resistance in bacteria, including staphylococci and enterobacteria. Like acridine orange, ethidium bromide is intercalated between base-pairs of DNA. It has previously been shown that ethidium bromide eliminates antibiotic resistance at a higher frequency than acridine dyes [232]. The method chosen was that described by Bouanchaud *et al.* [232], which is essentially similar to that for plasmid curing by acridine orange.

The experiment was performed as before. The growth of *A. baumannii* 6B92 was visibly inhibited by 64 mg/l of ethidium bromide. 100µl of nutrient broth was subcultured from this dilution onto antibiotic free medium (IST agar). Forty distinct colonies were tested for elimination of carbapenem resistance by subculturing onto IST agar and testing for imipenem resistance with a filter paper disc containing 10µg of imipenem. The percentage rate of cure was 10%.

Figure 19 presents a sensitivity plate inoculated with an imipenem resistant and sensitive colony of *A. baumannii* 6B92. A four fold increase in size of the zone of inhibition to imipenem, from 10 mm to 40 mm diameter was observed.

Figure 19.

Sensitivity plate inoculated with an imipenem resistant (right side) and sensitive (left side) colony of *A. baumannii* 6B92

4.10. API profile of the cured strain

To ensure the integrity of the cured strain, an API 20NE identification strip was set up. The API identification profile for both strains was identical.

4.11. Stability of loss of resistance to imipenem for the cured strain

To determine whether the loss of resistance to imipenem was permanent. The cured strain was grown overnight at 37°C in 10 ml of nutrient broth containing a sub-MIC amount of imipenem (0.06mg/l). A 20µl amount of this dilution was sub-cultured into the same imipenem containing broth over a 10 day period. A sensitivity plate was set up each day from the inoculated broth and an imipenem disc placed onto the agar. No resistant colonies were seen growing into the zone of inhibition of the imipenem disc

This suggests that the loss of resistance to imipenem was permanent.

The following conclusions may be made:

1. The genetic determinant of carbapenem resistance is located on a plasmid which is not possible to visualise or isolate.

or

2. The genetic determinant is chromosomally based and the curing experiments have effected a mutation in the host chromosome.

Either of these two statements are feasible, although the latter statement is unlikely and the genetic location of the resistance gene is probably on a plasmid.

To determine the extent of loss of resistance to imipenem, and to other antimicrobials, MICs and disc sensitivities were performed on the 'cured' strain.

4.12. Susceptibility testing of the imipenem sensitive (cured) strain of *A. baumannii* 6B92

Table 39 shows the MICs and disc sensitivities of the cured strain. The disc sensitivity tests demonstrated loss of resistance to all penicillins and carbapenems. This was further confirmed by MIC data, as MICs of penicillins were reduced by a factor of at least 8. The loss of resistance to the carbapenems was most marked, with the MIC of imipenem and meropenem reduced by a factor of 128. The MICs of second and third generation cephalosporins were not significantly reduced.

4.13. Isoelectric focusing of the cured strain of *A. baumannii* 6B92

The loss of resistance markers to penicillins and carbapenems but not to other classes of antimicrobials would strongly suggest that this resistance had been mediated by a ß-lactamase. To confirm whether there was any loss of ß-lactamase production by the cured strain, it was subjected to isoelectric focusing as before.

It can be clearly seen that the cured strain contained only the diffuse (presumed chromosomal) enzyme (figure 20). The ARI-1 ß-lactamase could not be visualised on IEF.

Table 39.

Disc susceptibility results and MICs of various antibiotics to both the parent strain and the cured strain

Antibiotic disc (μg)	6B92		6B92 (Cured strain)		Ratio of MIC of Parent strain / Cured strain
	Disc	MIC+	Disc	MIC+	
Ampicillin (10)	R	>256	I	16	16
Piperacillin (75)	R	-	I	-	
Azlocillin (75)	R	128	I	16	8
Amp/Clav* (30)	R	>128	I	8	8
Cephaloridine (30)	R	-	R	-	
Cephalexin (30)	R	-	R	-	
Cefuroxime (30)	R	32	R	32	1
Cefotaxime (10)	I	16	I	8	2
Ceftazidime (10)	I	4	I	4	1
Cefoxitin (10)	R	-	R	-	
Trimethoprim (2.5)	R	-	R	-	
Gentamicin (10)	S	0.5	S	0.5	1
Ciprofloxacin (1)	S	0.25	S	0.25	1
Imipenem (10)	R	16	S	0.12	128
Meropenem	-	32	-	0.25	128
Aztreonam (30)	R	-	R	-	

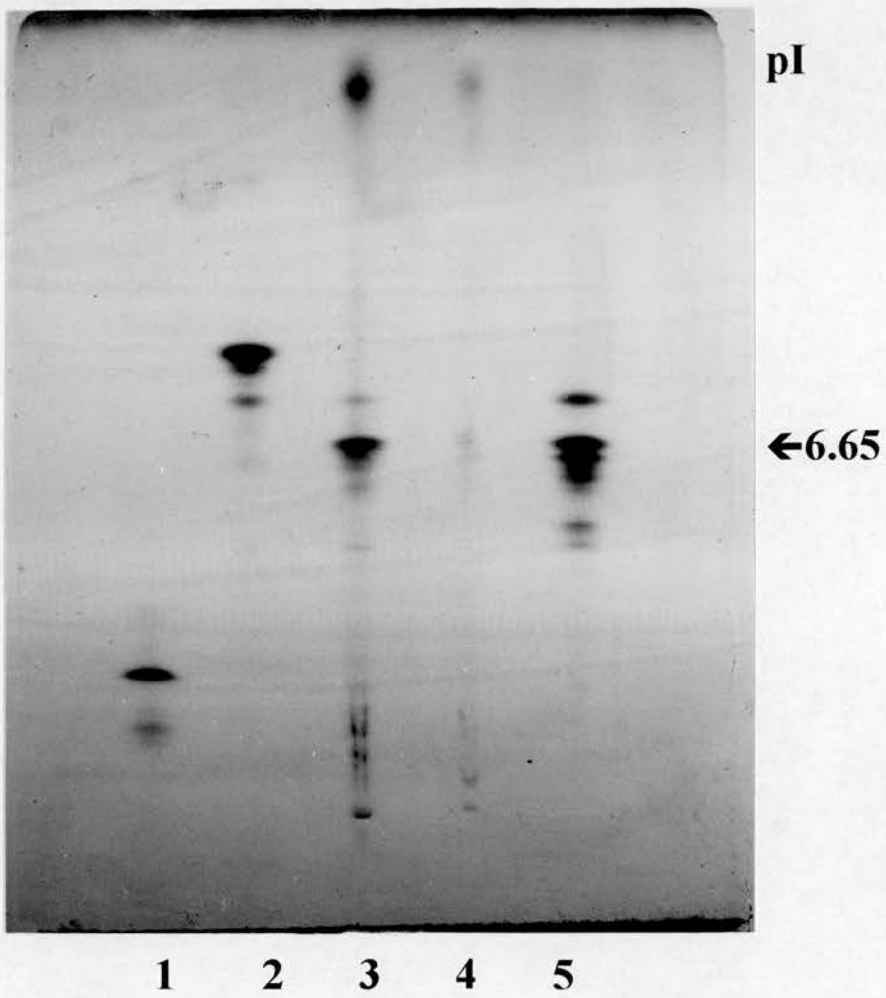
Abbreviations: R, resistant; I, intermediate; S, sensitive.

*2:1 ratio. The results are expressed in terms of ampicillin.

-, Not tested; +, MIC expressed as mg/l.

Figure 20.

Isoelectric focusing of *Acinetobacter baumannii* 6B92 ß-lactamases after curing experiments with ethidium bromide



IEF gel contained ampholines pH range 3.5-10.6

Lane 1: TEM-1 ß-lactamase (pI 5.4)

Lane 2: SHV-1 ß-lactamase (pI 7.6)

Lane 3: *Acinetobacter baumannii* 6B92 (parent strain)

Lane 4: *Acinetobacter baumannii* 6B92 (cured strain, ß-lactamase activity of the enzyme of pI>9.0 was stronger in other preparations)

Lane 5: *Acinetobacter baumanni* 6B92 (purified through G-75 sephadex column)

In conclusion:

This result corroborates the findings from the other experiments. Once cured of imipenem resistance with the subsequent loss of the ARI-1 ß-lactamase the strain displayed loss of resistance to penicillins and carbapenems. The loss of resistance was most apparent for carbapenems. The MICs of second and third generation cephalosporins were not significantly reduced suggesting that resistance to these ß-lactams came from the chromosomal ß-lactamase of high pI. There was still some residual resistance to the penicillins, and this may be as a result of hydrolysis by the chromosomal cephalosporinase. Carbapenems are not hydrolysed by Acinetobacter chromosomal ß-lactamases, and this would explain the more impressive loss of resistance to these compounds.

The microbiological assay was also consistent with the sensitivity data. The cured strain only displayed loss of resistance to those compounds that were inactivated by the ARI-1 ß-lactamase but not to those compounds to which inactivation or hydrolysis could not be demonstrated.

The evidence for carbapenemase activity by the ARI-1 ß-lactamase was almost complete. One nagging concern, however, was that the genetic determinant of carbapenem resistance could not be transferred to other species, that would allow the expression of the ARI-1 ß-lactamase to be examined in a well-characterised host. It could still be argued therefore, that changes in outer membrane permeability or alterations in PBPs could not be discounted and could contribute to carbapenem resistance.

The recent availability of BRL 42715, a suicide inhibitor of serine active site enzymes has provided us with a simple method for this purpose. A recent publication has successfully used this compound for evaluating the role of cephalosporinase activity in *Ps. aeruginosa* resistant to imipenem [233].

4.14. Sensitivity of *A. baumannii* 6B92 to the carbapenem/BRL 42715 combination

An MIC determination on both the cured and parent strain to imipenem and meropenem was performed, with the addition of a fixed concentration of BRL 42715 (4 mg/l) to all dilutions. An inoculum of 10^4 cfu/spot was applied to IST agar and the plates incubated overnight in air at 37°C.

The results are shown in table 40.

Table 40.

MIC values of *Acinetobacter baumannii* 6B92 to carbapenems, combined with the ß-lactamase inhibitor BRL 42715

	MIC (mg/l)	
Antibiotic	**6B92 (parent strain)**	**6B92 (cured strain)**
Imipenem	16.0	0.12
Imipenem + 4 mg/l BRL 42715	0.12	0.06
Meropenem	32.0	0.25
Meropenem + 4 mg/l BRL 42715	0.5	0.25

The results show that resistance to carbapenems by the parent strain has been eliminated by the addition of BRL 42715, the MICs have been reduced to almost the same level as the MICs of the cured strain.

This final piece of evidence confirms the role of the ARI-1 ß-lactamase to carbapenem resistance in *A. baumannii* 6B92.

5.0. The ß-lactamases of the species *Xanthomonas maltophilia*

5.1. Introduction

The species *X. maltophilia* was excluded from the initial survey of cefuroxime-resistant blood culture isolates because of its intrinsic resistance to this compound and many other antimicrobials. As mentioned in the introduction, this resistance has been attributed to the interplay between the production of a serine active site cephalosporinase (L2), a metallo-ß-lactamase (L1) and the impermeability of the outer membrane.

There has been contrasting information concerning the production of ß-lactamases in *X. maltophilia*. Two recent studies have shown an unexpected heterogeneity among ß-lactamases produced by the species. Cullmann and Dick [205] examined 20 different strains and identified 6 distinct ß-lactamases by virtue of their isoelectric point. None of these strains produced more than one ß-lactamase. They all demonstrated the ability to hydrolyse carbapenems. None of these enzymes was inhibited by EDTA, which suggests this carbapenem hydrolysing ability may not be conferred by a metallo-ß-lactamase. Mattionni *et al.* [204] reported similar data, with the demonstration of 5 ß-lactamases which also hydrolysed carbapenems and were again differentiated solely by virtue of their isoelectric points. None of these two latter surveys included strains which encoded for the production of the two chromosomal ß-lactamases (L1 and L2), described originally by Saino *et al.* [123,203].

Recent studies have also shown that MICs of ß-lactam antibiotics for *X. maltophilia* isolates vary with the test medium employed [226-228].

The MIC determinations of the *X. maltophilia* isolates from the blood culture susceptibility survey strains were originally performed on either DST or IST agar, dependant on when they were isolated, and to what ß-lactams they were tested against. This generates a degree of uncertainty as to the validity of the previous MIC work. For this reason, it was decided to re-examine these strains to determine any difference in susceptibility to various antimicrobials on differing media. IST and Mueller-Hinton agar are two of the most commonly used sensitivity test media, and were therefore selected for use in this study. Some of these isolates were from the

same patients but isolated at differing time intervals and were probably different strains and not repeat isolates, they were therefore included in the survey. Strains isolated in 1992, were also included to allow a larger population of *X. maltophilia* to be examined.

The aim of this part of the study was therefore twofold:

- To investigate the incidence and level of antimicrobial resistance of the species on differing susceptibility test media. This would provide a clearer insight into the susceptibility of these strains, and hopefully provide a guidance as to which medium was the more suitable for susceptibility testing of this species.

- To attempt to clarify the production of ß-lactamases in this species, which at best can presently be described as confused, with particular attention to their role in carbapenem resistance.

5.2. Antimicrobial susceptibilities of *X. maltophilia* isolates

The MIC determinations of the test strains were performed as described in Materials and Methods. The test media employed were IST agar and MH agar. The results are shown in tables 41 to 42.

In general, most strains were resistant to all antimicrobials tested, although there were major differences in the MICs of many strains, dependant on the media employed. Generally, the isolates were more sensitive to the ß-lactams when MICs were performed on IST agar, in many cases a four-fold decrease in resistance was observed when compared to the values obtained for those on MH agar. The only ß-lactam to exhibit similar susceptibility values on both media was cefuroxime. All strains tested exhibited MIC levels of >256mg/l to this compound. On the other hand, MIC values for other classes of antimicrobials, such as gentamicin and ciprofloxacin on the two media were more in agreement, although some significant discrepancies to these agents were also observed on the different media. Strains 8B368, 8B376, 8B379 and 8B404, were all isolated from the same patient over approximately a one month duration. The MIC values for each of these strains were different. Specifically, 8B376 was less resistant to most of the antimicrobials tested, particularly to gentamicin and ciprofloxacin.

This result has serious implications for the clinical laboratory. If breakpoint values were to be used, in some cases, strains tested on IST agar would have been reported as sensitive to certain ß-lactams, whereas on MH agar these strains would have been designated as resistant. The nature of this anomaly has been investigated by other workers, and will not be examined further in this thesis.

Table 41.

MICs (mg/l) of the *X. maltophilia* isolates against various antibiotics

	Ampicillin		Piperacillin		Azlocillin		Cefuroxime		Cefotaxime		Ceftazidime	
Strain	IST	MH	IST	MH	IST	MH	IST	MH	IST	MH	IST	MH
5B105	64	>256	16	>256	16	>256	>256	>256	16	128	8	>256
6B52	64	>256	16	128	4	16	>256	>256	16	128	8	>256
6B133	128	>256	32	>256	16	>256	>256	>256	16	128	8	>256
6B295	64	>256	16	>256	32	>256	>256	>256	16	128	16	>256
7B78	16	>256	8	64	4	16	>256	>256	1	64	4	128
8B368	128	>256	32	>256	16	64	>256	>256	16	16	4	8
8B376	16	64	16	16	2	16	>256	>256	1	8	1	8
8B379	64	128	64	>256	16	32	>256	>256	16	16	2	8
8B404	16	32	16	64	16	16	>256	>256	4	8	1	4
8B405	32	>256	16	128	16	32	>256	>256	32	128	4	64
9B123	64	256	4	64	4	16	>256	>256	8	32	1	8
9B266	128	>256	4	64	4	64	>256	>256	8	32	2	8
10B6	128	>256	32	128	16	32	>256	>256	32	64	4	64
10B44	32	>256	32	>256	16	64	>256	>256	4	32	1	16
10B411	32	>256	8	64	8	32	>256	>256	2	16	4	32
11B4	32	>256	16	128	8	32	>256	>256	4	32	2	16
11B29	128	>256	32	128	16	32	>256	>256	32	64	4	64
12B286	128	>256	16	128	4	32	>256	>256	16	128	8	128
12B346	64	>256	16	256	16	256	>256	>256	32	256	4	>256
12B457	1	>256	16	64	4	16	>256	>256	8	64	2	16
12B507	128	>256	16	>256	16	256	>256	>256	128	256	8	>256
13B35	128	>256	32	128	16	64	>256	>256	16	64	4	16
13B318	4	4	16	64	2	4	>256	>256	4	16	1	2
13B275	>256	>256	16	128	4	32	>256	>256	16	64	2	8
13B289	>256	>256	16	128	4	16	>256	>256	16	64	2	8
13B344	>256	>256	16	128	4	16	>256	>256	16	128	8	64
13B357	>256	>256	16	128	8	16	>256	>256	16	128	8	64
13B366	>256	>256	16	128	4	16	>256	>256	16	128	8	64

IST; Isosensitest agar, MH Mueller Hinton agar.

Table 42.

MICs (mg/l) of the *X. maltophilia* isolates against various antibiotics

Strain	Ciprofloxacin		Meropenem		Imipenem		Gentamicin		Aztreonam	
	IST	MH	IST	MH	IST	MH	IST	MH	IST	MH
5B105	4	4	32	128	16	256	64	64	16	>256
6B52	8	8	32	128	64	128	64	128	64	>256
6B133	8	8	32	256	64	>256	64	128	64	>256
6B295	4	4	32	128	16	256	64	64	16	>256
7B78	4	4	4	64	4	256	2	4	64	256
8B368	32	32	64	64	16	256	4	16	64	>256
8B376	2	0.5	4	64	4	64	0.5	2	8	16
8B379	64	64	8	16	32	256	2	16	32	32
8B404	64	32	4	8	4	16	1	16	16	16
8B405	32	32	64	256	32	256	16	2	32	>256
9B123	8	4	32	128	16	256	64	64	32	>256
9B266	8	2	32	128	64	256	64	128	128	256
10B6	32	8	64	256	128	256	64	128	32	256
10B44	32	16	2	16	8	128	16	64	32	>256
10B411	4	2	16	128	16	256	16	4	16	32
11B4	16	8	2	32	4	128	16	2	64	256
11B29	32	8	64	256	128	256	64	2	32	>256
12B286	8	4	32	256	64	64	64	64	16	>256
12B346	8	2	32	256	16	128	64	32	32	>256
12B457	16	8	8	32	16	256	16	16	32	>256
12B507	4	2	32	256	32	>256	16	4	128	>256
13B35	32	16	16	128	16	256	64	64	32	64
13B318	8	4	4	8	64	64	64	32	32	128
13B275	8	16	2	8	32	64	16	32	32	>256
13B289	8	4	2	8	64	64	16	32	32	>256
13B344	16	16	64	128	64	256	64	256	128	>256
13B357	8	4	64	256	32	256	64	256	128	>256
13B366	8	16	64	128	64	256	64	256	128	256

IST; Isosensitest agar, MH; Mueller Hinton agar

6.0. Characterisation of *X. maltophilia* ß-lactamases

Initially, to investigate the ß-lactamases from *X. maltophilia* it was decided to employ isoelectric focusing. This is an easy technique to perform, and would primarily allow the detection of any obvious differences in the ß-lactamase complement of this species.

6.1. Preparation of ß-lactamases

All 28 isolates of *X. maltophilia* were examined. Initial studies on the ß-lactamases from this species were performed on small scale ß-lactamase preparations. The activity of these preparations, as measured by the nitrocephin spot test, was found to be very poor, with reaction times of greater than 30s. Subsequent analytical IEF of these preparations was disappointing, with few strains exhibiting any detectable ß-lactamase activity (data not shown). Therefore, in an attempt to increase the yield of ß-lactamase produced, the isolates were subjected to induction of ß-lactamase prior to extraction of the enzyme.

6.2. Induction of small scale ß-lactamase preparations

Induction of ß-lactamase was as described in Materials and Methods. Cefotaxime, which has previously been shown to be a potent inducer of *X. maltophilia* ß-lactamases [228] was added at ¼ the MIC to the broth.

After induction of the strains under test, ß-lactamase activity was found to be greatly enhanced with the nitrocephin spot test reaction times for all isolates reduced to between 1 and 5 seconds.

This result indicates that all ß-lactamase production in these strains was inducible.

6.3. Analytical isoelectric focusing

Induced samples were applied to a polyacrylamide gel containing ampholines with a pH range 3.5-10.6.

6.3.1. Results

Figures 21 to 23 show the result of the isoelectric focusing. Initial staining of the gel with nitrocephin appeared to show the production of a single ß-lactamase, usually of basic pI, from each strain. The exception to this was a ß-lactamase(s) that focused as a series of bands of activity ranging from pI 5.2-6.8. This focusing pattern was the most common among the survey strains and was present in seven of the 28 strains examined (5B105; 6B52; 6B133; 6B295; 7B78; 12B286; 12B346).

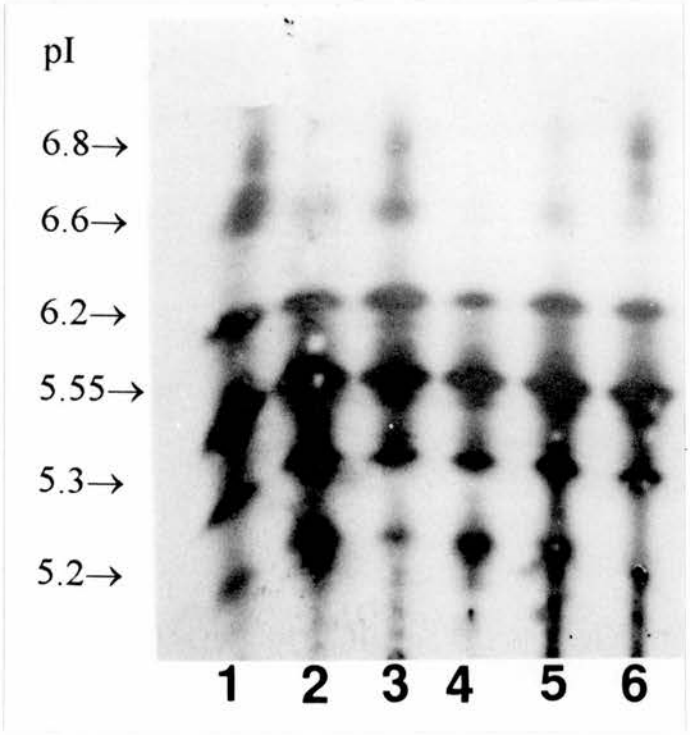
These results would have been consistent with the findings of Cullmann *et al.* [205], who also recognised a single ß-lactamase in all of their strains. However, in this study, it was observed that following prolonged staining, other bands of ß-lactamase activity were visualised in most strains, generally at lower pIs than the initial ß-lactamase. The pI values are listed in Table 43. It was assumed that the increased time for these enzymes to be visualised was as a result of their lower hydrolytic activity against nitrocephin.

Strains 13B275 and 13B289 exhibited identical IEF patterns. These were isolated from the same patient, but at different times. Strains 13B344, 13B357 and 13B366 also shared identical isoelectric focusing patterns as one another, 13B344 and 13B366 were from the same patient, 13B357 was isolated from another source. The strains 8B368, 8B376, 8B379 and 8B404 that were from a single patient, shared a common ß-lactamase profile, aside from 8B376 which only harboured the ß-lactamase of pI 9.7, but not the two other bands of pI 5.4 and 4.95 seen in the other three strains. Strain 8B376 was less resistant to many of the antibiotics examined than the other isolates from this patient, which may in part be as a result of the loss of these particular ß-lactamases, although it should be noted that both gentamicin and ciprofloxacin resistance was also diminished in this strain, indicating the inclusion of other resistance mechanisms. The ß-lactamase profile of strains 10B6 and 11B29 were identical, these were from different patients.

These results not only indicate a high level of heterogeneity of ß-lactamase production in different isolates of *X. maltophilia*, but also the presence of multiple ß-lactamases in these strains. The most common ß-lactamase profile focused as a series of bands of activity ranging from pI 5.2-6.8. This pattern was displayed by seven of the study strains (table 43). All these strains were isolated from different patients over the entire study period, and were therefore unlikely to be duplicate isolates.

Figure 21.

Isoelectric focusing of ß-lactamases from *X. maltophilia* blood culture isolates



IEF gel contained ampholines pH range 3.5-10.6

Lane 1: *X. maltophilia* 5B105
Lane 2: *X. maltophilia* 6B52
Lane 3: *X. maltophilia* 6B133
Lane 4: *X. maltophilia* 6B295
Lane 5: *X. maltophilia* 7B78
Lane 6: *X. maltophilia* 12B286

Figure 22.

Isoelectric focusing of ß-lactamases from *X. maltophilia* blood culture isolates

IEF contained ampholines pH range 3.5-10.6

Lane	1:	*X. maltophilia* 8B368	Lane	2:	*X. maltophilia* 8B376
Lane	3:	*X. maltophilia* 8B379	Lane	4:	X. *maltophilia* 8B404
Lane	5:	TEM-1 (pI 5.4)	Lane	6:	SHV-1 (pI 7.6)
Lane	7:	*X. maltophilia* 8B405	Lane	8:	*X. maltophilia* 9B123
Lane	9:	*X. maltophilia* 9B266	Lane	10:	*X. maltophilia* 10B6
Lane	11:	*X. maltophilia* 13B275	Lane	12:	*X. maltophilia* 13B289

Figure 23.

Isoelectric focusing of ß-lactamases from *X. maltophilia* blood culture isolates

IEF contained ampholines pH range 3.5-10.6

Lane	1:	*X. maltophilia* 10B44	Lane	2:	*X. maltophilia* 10B411
Lane	3:	*X. maltophilia* 11B4	Lane	4:	X. *maltophilia* 11B29
Lane	5:	X. *maltophilia* 12B346	Lane	6:	*X. maltophilia* 12B457
Lane	7:	*X. maltophilia* 12B507	Lane	8:	*X. maltophilia* 13B35
Lane	9:	*X. maltophilia* 13B318	Lane	10:	*X. maltophilia* 13B344
Lane	11:	*X. maltophilia* 13B357	Lane	12:	*X. maltophilia* 13B366

Table 43.

Isoelectric points of ß-lactamases from *X. maltophilia*

Strain number	Isoelectric points of ß-lactamases
5B105	6.8, 6.2, 5.55, 5.3
6B52	6.8, 6.2, 5.55, 5.3
6B133	6.8, 6.2, 5.55, 5.3
6B295	6.8, 6.2, 5.55, 5.3
7B78	6.8, 6.2, 5.55, 5.3
8B368	9.7, 5.4, 4.95
8B376	9.7
8B379	9.7, 5.4, 4.95
8B404	9.7, 5.4, 4.95
8B405	9.3, 6.2
9B123	7.8, 6.9
9B266	9.7
10B6	6.7, 6.3
10B44	9.2, 5.9
10B411	9.4, 6.3
11B4	10.0, 5.5
11B29	6.7, 6.3
12B286	6.8, 6.2, 5.55, 5.3
12B346	6.8, 6.2, 5.55, 5.3
12B457	9.7, 8.2
12B507	10.1
13B35	9.3, 6.1
13B318	10.0, 6.9, 5.4
13B275	9.0, 6.2, 5.3
13B289	9.0, 6.2, 5.3
13B344	9.6, 6.2
13B357	9.6, 6.2
13B366	9.6, 6.2

6.4. Conjugation experiments

Conjugation experiments were performed on all strains. These conjugation experiments were unsuccessful. Despite varying the incubation times and temperature of the conjugations, no transconjugants were detected.

As discussed previously, it would appear that all ß-lactamase production in this species was inducible, and hence unlikely to be plasmid-mediated. These enzymes were therefore assumed to be of chromosomal origin.

From these results, it was possible to portray at least twelve differing isoelectric focusing patterns on IEF gels. These were thus designated as ***Xanthomonas maltophilia* chromosomal enzymes** (XMCEs), and a tentative classification scheme is shown in table 44, based upon the pI values of these enzymes.

6.5. Conclusions

This study has shown the wide diversity of chromosomal ß-lactamases produced by *X. maltophilia*, with some strains demonstrating multiple bands of activity.

The methodology employed in this study differentiated these enzymes solely on the basis of their isoelectric point. Categorising ß-lactamases by isoelectric point alone, yields no information regarding substrate and inhibitor profiles, and therefore provides insufficient criteria to determine whether these enzymes have diverse biochemical properties.

Detailed biochemical investigations are essential to elucidate the full nature of these enzymes.

XMCE type-1 was the most universal enzyme type detected, and may be a common ß-lactamase pattern among isolates of *X. maltophilia*. Strain 5B105 was selected as representative of the XMCE type-1 group, for detailed biochemical investigations.

Table 44.

Classification scheme for *X. maltophilia* chromosomal ß-lactamases

XMCE* type	Strain (s)	pI(s)
1	6B105, 6B52, 6B133, 6B295, 7B78, 12B286, 12B346	6.8, 6.2, 5.55, 5.3
2	8B368, 8B379, 8B404	9.7, 5.4, 4.95
2a	12B457	9.7, 8.2
2b	8B376, 9B266	9.7
3	8B405	9.3, 6.2
4	9B123	7.8, 6.9
5	10B6, 11B29	6.7, 6.3
6	10B44	9.2, 5.9
7	10B411	9.4, 6.3
8	13B318	10.0, 6.9, 5.4
8b	11B4	10.0, 5.5
9	12B507	10.1
10	13B35	9.3, 6.1
11	13B275, 13B289	9.0, 6.2, 5.3
12	13B344, 13B 357 13B366	9.6, 6.2

XMCE*, *Xanthomonas maltophilia* chromosomal enzyme.

7.0. Purification and biochemical characterisation of the XMCE type-1 *X. maltophilia* ß-lactamase 5B105

The biochemical characterisation was carried out as recommended by Bush and Sykes [171].

7.1. Assessment of inducibility of ß-lactamase

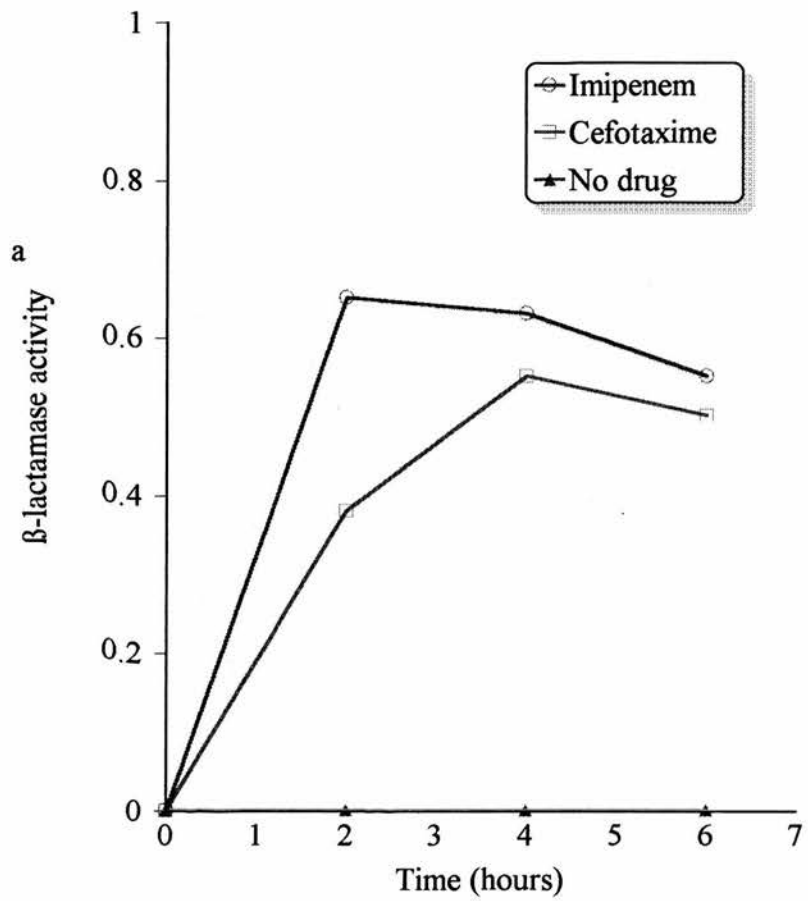
Either cefotaxime or imipenem was employed as inducer, previous studies have shown both these compounds to be strong inducers of *X. maltophilia* ß-lactamases [204,226]. They were added at ¼ the MIC. Nitrocephin (50mg/l) was used as the reporter substrate. Protein estimation was measured by the method of Waddell [218].

The kinetics of ß-lactamase induction for strain 5B105 are shown in figure 24. When imipenem or cefotaxime was added as inducer, ß-lactamase activity increased sharply after less than one hour. Imipenem was a more potent inducer of ß-lactamase than cefotaxime. No discernible ß-lactamase activity was detected in disrupted cells without the prior addition of inducer.

This result confirms that all ß-lactamase activity was inducible. No enzyme activity was detected in the culture filtrate, indicating that the location of ß-lactamase was intracellular.

Figure 24.

Kinetics of ß-lactamase induction of *X. maltophilia* 5B105



a, Log_{10} nanomoles nitrocephin hydrolysed/min/mg of protein.

7.2. Large scale preparation of ß-lactamases

Imipenem was used as the inducer for all large scale preparations.

7.3. Isoelectric focusing

We have previously shown the presence of at least four main bands commensurate with major ß-lactamase activity (pI 6.8, 6.2, 5.55, 5.3) with two minor bands (5.2, 6.6) produced by strain 5B105.

In their guidelines for the evaluations of novel ß-lactamases [171], Bush and Sykes recommended that characterisation be performed with a homogeneous enzyme preparation, or a minimum requirement would be the demonstration of a single ß-lactamase activity on isoelectric focusing. *X. maltophilia* 5B105 presents several bands of activity, clearly the next stage was to determine whether these bands were satellite bands produced by a single enzyme, or several discrete ß-lactamases.

7.3.1. Isoelectric focusing with ß-lactamase inhibitor overlays

To further elucidate the nature of these ß-lactamases it was decided to employ a technique originally described by Bakken *et al.* [234] for the tentative characterisation of ß-lactamases from *Aeromonas* spp. This procedure involved overlaying an IEF gel with filter paper strips soaked with various ß-lactamase inhibitors; this easy to apply technique might allow an initial distinction between these ß-lactamases.

To allow the effect of the ß-lactamase inhibitors on the bands of ß-lactamase activity to be studied with greater clarity, we endeavoured to improve the resolution of these enzymes. A customised gel was prepared. This gel contained a 1:1 ratio of ampholines with a pH range of 3.5-10.6 and 4-6. This would create a non-linear pH gradient, but would increase the resolution of the ß-lactamase bands in the pI 4-6 range.

The inhibitors were chosen for their selective action on specific classes of ß-lactamases.

The inhibitors and concentrations used were:

1 1mM EDTA, metal ion chelator, known to inhibit metallo-ß-lactamases.
2 1mM potassium clavulanate, an inhibitor of penicillinases and broad spectrum ß-lactamases.
3 1mM aztreonam, an inhibitor of cephalosporinases.
4 100µM BRL 42715, an inhibitor of all serine active site ß-lactamases, but not metallo-ß-lactamases.
5 Cloxacillin, an inhibitor of cephalosporinases.

Isoelectric focusing was performed as before, the gels were stained with nitrocephin after a 30 second overlay with the various inhibitors soaked in filter paper. A control lane was established with no prior overlay of inhibitor. The results are shown in figure 25.

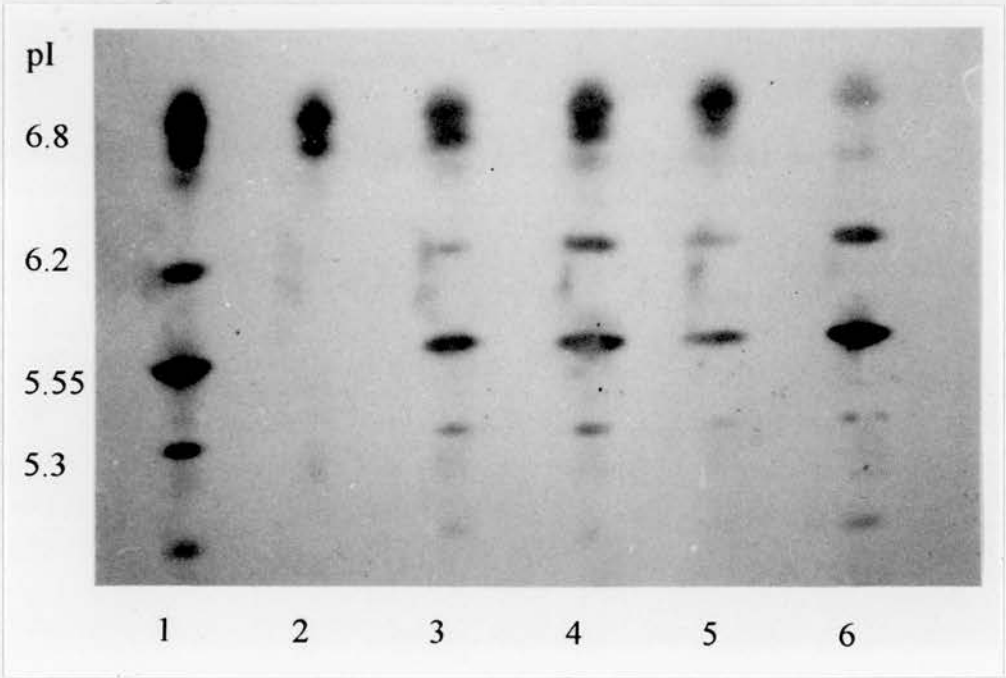
The ß-lactamase band of pI 6.8 was partially inhibited by the overlay of EDTA prior to staining, whereas all other bands were unaffected. Conversely, the other ß-lactamase bands were completely inhibited by the overlay of BRL 42715 and partially inhibited by potassium clavulanate. Aztreonam and cloxacillin overlays appeared to have no significant effect on any of the ß-lactamase bands.

The inhibitor overlays proved to be a useful tool, although it should be noted that the results are qualitative, and no substitute for formal quantitative inhibitor profiles. The following assumptions could be drawn: The band of activity of pI 6.8 was partially inhibited by EDTA and may be a metallo-ß-lactamase.The other three main bands (6.2, 5.55, 5.3) were inhibited by BRL 42715 and potassium clavulanate. This result would intimate that they were serine active site penicillinases or broad-spectrum ß-lactamases, non inhibition by cloxacillin or aztreonam would infer that they were unlikely to be cephalosporinases.

We have therefore demonstrated by a simple technique that *X. maltophilia* 5B105 encoding for XMCE type-1 produces at least 2 and possibly more ß-lactamases. The

Figure 25.

Isoelectric focusing patterns of *X. maltophilia* 5B105 ß-lactamases overlaid with various inhibitors



IEF gel contained a 1:1 ratio of ampholines pH range 4-6 / 3.5-10.6

Lane 1: control

Lane 2: overlaid with 100µM BRL 42715

Lane 3: overlaid with 1mM aztreonam

Lane 4: overlaid with 1mM cloxacillin

Lane 5: overlaid with 1mM clavulanate

Lane 6: overlaid with 1 mM EDTA.

next stage was to purify these enzymes to enable their individual substrate and inhibitor profiles to be examined in more detail.

7.4. Purification of ß-lactamases by low pressure Sephadex G-75 gel filtration chromatography

Two ml of a large scale cell-free extract prepared from 1L of MH broth, previously induced with imipenem at ¼ the MIC, was applied to a Sephadex G-75 gel filtration column, previously equilibrated with 50 mM Tris Cl buffer (pH 8.0) containing 0.1 mM zinc sulphate. The sample was eluted with the same buffer with a flow rate of 12 ml/hour. ß-lactamase activity was detected with nitrocephin as before.

7.4.1. Results

Most of the ß-lactamase activity was detected in the fractions containing the void volume. The G-75 gel matrix has a useful fractionation range of 1×10^3 - 5×10^4 daltons. This result demonstrates that these enzymes are of large M_r (≥50kDa).

The apparent large molecular weight of these enzymes, might indicate these ß-lactamases exist in sub-unit form. This would not be unexpected, for previous ß-lactamases from these species have been shown to exist in sub-unit form [124,203].

7.5. Gel filtration on Sephadex G-150 gel column

The next stage was to attempt purification by employing a gel matrix with a wider fractionation range. The G-150 Sephadex gel matrix was chosen. This gel has a M_r fractionation range of up to 1.5×10^5 daltons.

The column was calibrated with the molecular weight standards alcohol dehydrogenase (150000), bovine serum albumin (66000) and cytochrome C (12384). The samples were eluted at the same rate of flow and with the same buffer used for the G-75 column. Any ß-lactamase activity present was initially identified with the nitrocephin spot test. Fractions displaying ß-lactamase activity were then assayed spectrophotometrically employing either nitrocephin (100µM) or imipenem (1mM) as reporter substrates. This would provide the following information:

Nitrocephin hydrolysis would indicate total ß-lactamase activity (assuming all ß-lactamases present hydrolysed this compound). Imipenem hydrolysis would indicate the presence of any carbapenemase activity.

7.5.1. Results

All ß-lactamase activity was confined within the molecular weight fractionation range of the column. Spectrophotometric analysis of the fractions eluted from the G-150 column (figure 26) and subsequent isoelectric focusing (figure 27), confirmed the presence of at least two distinct enzymes. The ß-lactamase with a pI of 6.8 eluted first from the column and hydrolysed imipenem as well as nitrocephin, the other ß-lactamase bands eluted from the column at the same rate, and hydrolysed nitrocephin but not imipenem.

Figure 26

Relative rate of hydrolysis of imipenem and nitrocephin by ß-lactamases from *X. maltophilia* 5B105 eluted from Sephadex G-150 gel filtration column

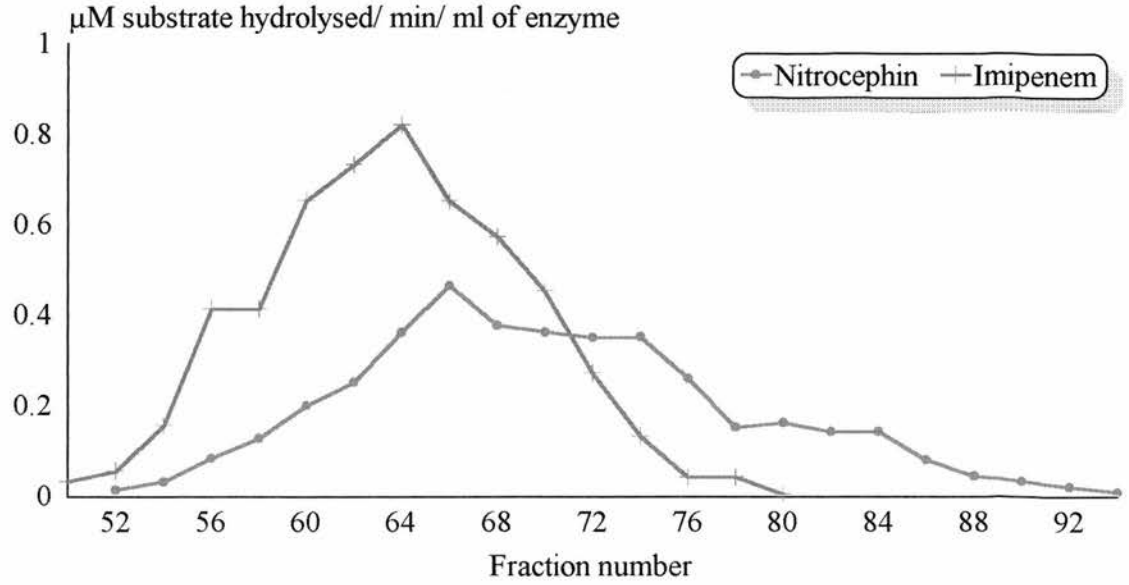
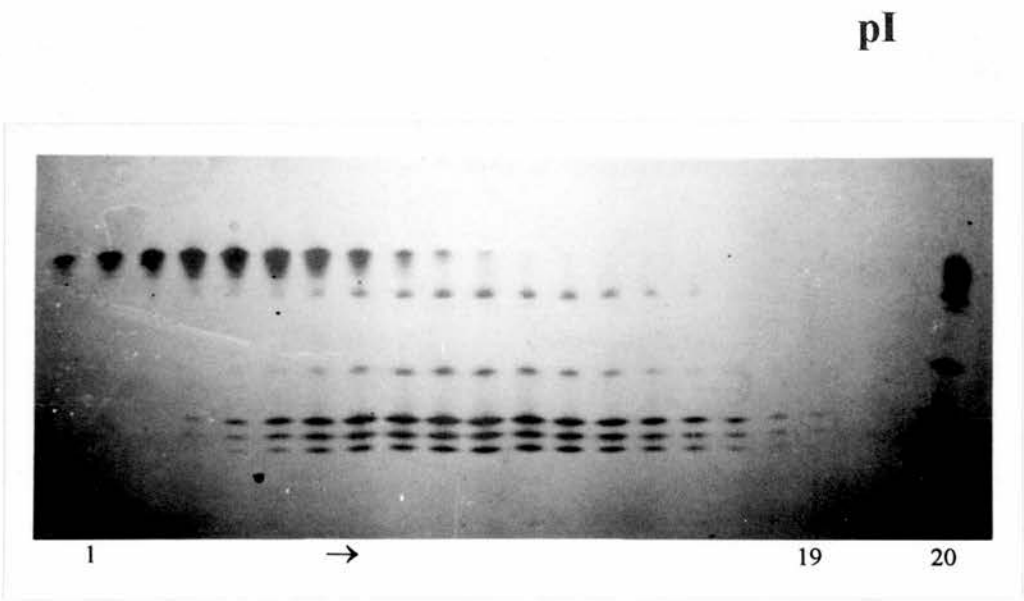


Figure 27.

Isoelectric focusing patterns of ß-lactamases from *X. maltophilia* 5B105 eluted from Sephadex G-150 column



IEF gel contained ampholines pH range 3.5-9.5

Lanes 1-19: Fifteen µls of every second fraction eluted from G-150 Sephadex column (fractions 52-88) applied to the gel.

Lane 20: Concentrated ß-lactamase preparation of *X. maltophilia* 5B105.

7.5.2. Molecular mass of ß-lactamases

By comparison of peak activity with the standard proteins [216].The M_r of the ß-lactamase of pI 6.8 was calculated as 96 kDa, whereas the M_r of the ß-lactamases of pI <6.8 was 48kDa.

7.5.3. Conclusions

The previous result affirms the presence of at least two ß-lactamases, both of high M_r. This suggests that these enzymes may have aggregated and exist in sub-unit form. We have also ascertained that the ß-lactamase of pI 6.8 is a carbapenem hydrolysing ß-lactamase, and is probably a metallo-ß-lactamase as a result of its inhibition by EDTA. The other ß-lactamase(s) present a more difficult problem. They appear to share a similar inhibitor profile and elute from the gel filtration column at the same rate, suggesting similar M_r.

Proteins can generally be separated by two components; their M_r and by their charge. We have shown that in their native form, the ß-lactamases of pI <6.8 have similar M_r. Therefore, it would seem logical to attempt to separate these enzymes by their charge (isoelectric point). Before we could do this we had to ascertain whether these multiple bands of ß-lactamase activity were satellite bands emanating from a single enzyme, or whether they were distinct ß-lactamases.

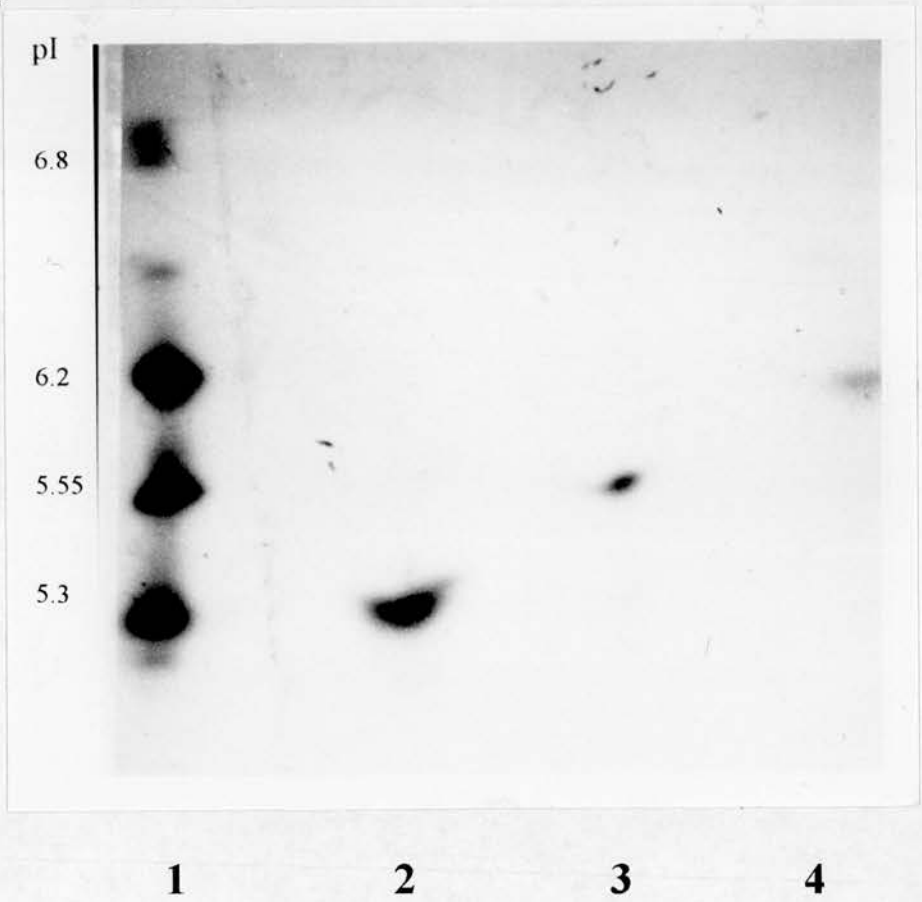
7.6. Purification of ß-lactamases by electrodialysis

Payne [235], demonstrated that when a ß-lactamase satellite band was excised from a gel, re-purified by electrodialysis and applied to an IEF gel and re-separated, the satellite band migrated to the original pI of the ß-lactamase, and not to the pI from which it was excised.

This technique was applied to the ß-lactamases of pI <6.8. The technique used was that described by Payne *et al.* [236]. The results are shown in figure 28.

Figure 28.

Isoelectric focusing of ß-lactamases from *X. maltophilia* 5B105 purified by electrodialysis



IEF gel contained a 1:1 ratio of ampholines pH range 4-6 / 3.5-10.6

Lane 1: Original ß-lactamase preparation from 5B105

Lanes 2-4: ß-lactamases bands (5.3, 5.55, 6.2) purified by electrodialysis.

7.6.1. Results

After separation by IEF and subsequent staining with nitrocephin, each preparation focused as a single band at exactly the same pI it had originally migrated to, indicating that each ß-lactamase was a discrete ß-lactamase rather than satellite banding.

The small yield of enzyme made this technique unsuitable for purification of enzyme for further studies.

This work has confirmed that the XMCE type-1 ß-lactamase profile from *X. maltophilia* 5B105 is comprised of several discrete ß-lactamases. Therefore, the next stage was to obtain enough pure enzyme solution of individual ß-lactamases to allow further studies on their properties.

Initially, it was decided to use the following technique:

> High performance ion exchange chromatography employing the Fast Protein liquid Chromatography (FPLC®) (Pharmacia) system.

Hopefully this would provide us with enough pure ß-lactamase solution of each enzyme to perform detailed M_r experiments, substrate and inhibitor profiles.

7.7. FPLC of the ß-lactamases from *X. maltophilia* 5B105

7.7.1. Methodology

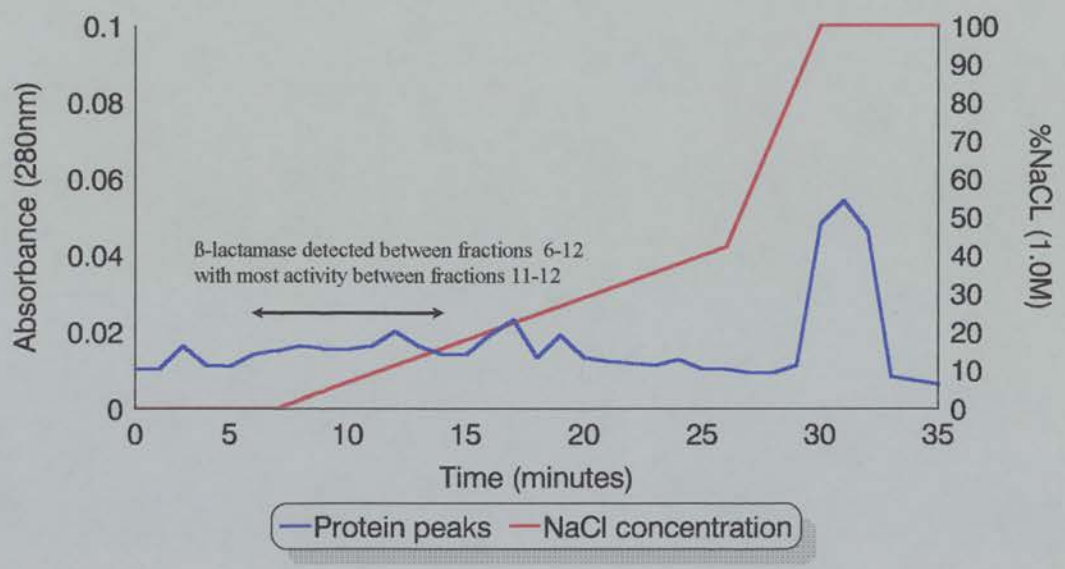
The fractions eluted from the G-150 column containing significant ß-lactamase activity were pooled (80ml) and concentrated to 5 ml in a Centriprep® 10 concentrator with a nominal molecular mass limit of 10kDa. One ml of the extract (5.44 mg protein) was applied to a Mono Q (HR 5/5) prepacked anion exchange column, previously equilibrated with 20 mM Tris Cl (pH 8.0) buffer containing 0.1 mM zinc sulphate. After washing, the elution was carried out with a continuous linear gradient of NaCl (1.0M) with a flow rate of 1 ml/min. Thirty-four 1ml fractions were collected. Each fraction was probed for ß-lactamase activity with the nitrocephin spot test.

7.7.2. Results

The trace of the FPLC separation of protein fractions is shown in figure 29.

Figure 29.

FPLC® trace of high performance anion exchange (Mono Q) of the partially purified ß-lactamases from *X. maltophilia* 5B105

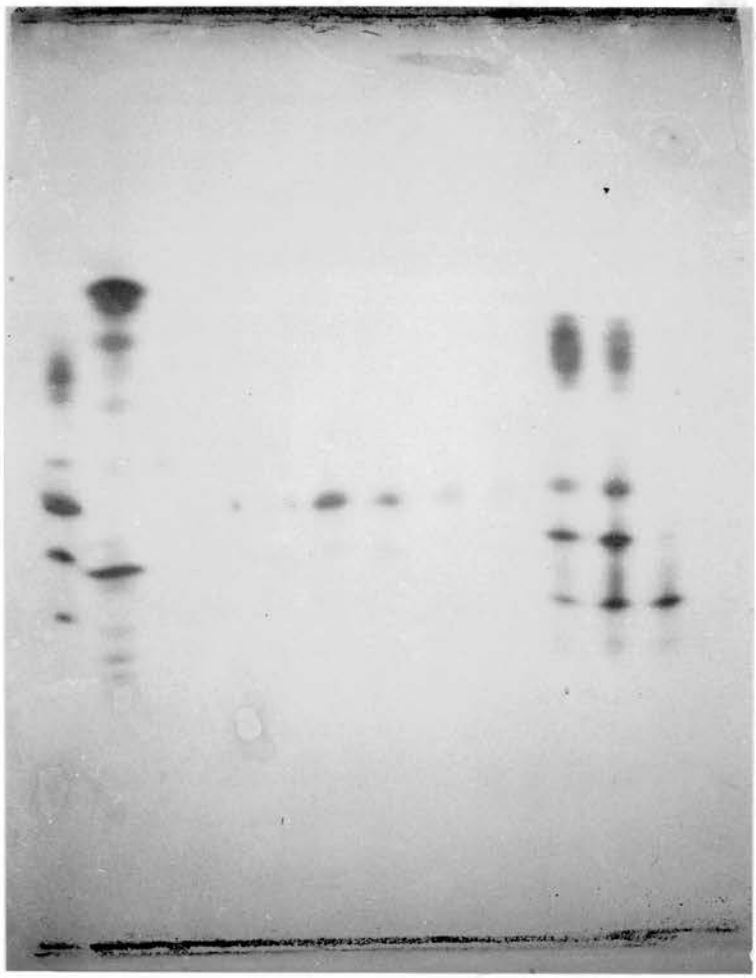


ß-lactamase activity was detected within fractions 4-12, although very small or no protein peaks were visible on the trace at these areas. Biological activity of all fractions was weak with nitrocephin spot test times of >30 seconds.

The fractions were applied to an IEF gel as before to determine whether the different ß-lactamase bands were successfully separated. The IEF is shown in figure 30. The pIs of the ß-lactamase(s), and protein concentrations contained in those fractions are shown in table 45.

Figure 30.

Isoelectric focusing of ß-lactamases from *X. maltophilia* 5B105 eluted from anion exchange step



1 2 3 4 5 6 7 8 9 10 11 12

IEF gel contained ampholines pH range 3.5-10.6

Lane 1: Original ß-lactamase preparation

Lane 2: TEM-1 and SHV-1 ß-lactamases

Lane 3: fraction 2 Lane 4: fraction 3 Lane 5: fraction 4

Lane 6: fraction 5 Lane 7: fraction 6 Lane 8: fraction 7

Lane 9: fraction 8 Lane 10: fraction 9 Lane 11: fraction 10

Lane 12: fraction 12

Table 45.

Isoelectric points of ß-lactamases eluted from anion exchange step

Fraction number	Isoelectric point of ß-lactamases present	Protein concentration of fraction (mg/ml)
4	none	-
5	6.2	-
6	6.2	0.21
7	6.2	-
8	6.2	-
9	6.2	-
10	5.3, 5.55, 6.2, 6.8	-
11	5.3, 5.55, 6.2, 6.8	0.297
12	5.3	0.067

-; not determined

The IEF gel shows that the ß-lactamase of pI 6.2 eluted first from the column between fractions 5 and 9 with peak activity (as ascertained by the intensity of staining on IEF) present in fraction 6. Fractions 10 and 11 contained all ß-lactamases that were present in the original preparation. Fraction 12 contained the ß-lactamase of pI 5.3.

7.7.3. Conclusions

The anion exchange step has succeeded in separating two of the four ß-lactamase bands. It should be noted, however, that the ß-lactamase of pI 6.2 that eluted first from the column may have comprised part of the initial void volume, as the salt gradient was not initiated until fraction 7. This would indicate that the enzyme has failed to bind to the column and has passed straight through along with other contaminating protein. Secondly, the ß-lactamase of pI 6.8 might have been expected to have eluted first as it holds the weakest net negative charge.

A concern at this stage was poor recovery and the weak activity of the purified ß-lactamases. Although the fractions remained stable when stored at -20^{o}C. The weak activity was either as a result of binding to the column matrix or removal and dilution of the proteins from their normal cellular environment.

7.8. Attempts to improve biological activity

To attempt to increase the activity of the eluted fractions, bovine serum albumin was added (to a final concentration of 1%) to each fraction after elution. This failed to enhance the biological activity of the enzyme.

We thought that the buffer system used may not be suitable and a Mono S cation exchange column equilibrated with 50 mM sodium acetate (pH 5.0) buffer was employed but this was found to have an even greater detrimental effect on the ß-lactamase activity and was not pursued any further.

Despite repeated attempts, the separations obtained with the Mono Q (HR 5/5) were the best that could be obtained. These fractions were used for further study of the properties of these ß-lactamases.

7.9. Native polyacrylamide gel electrophoresis (PAGE) on the PhastSystem®

Native PAGE separates proteins on the basis of their size, net charge and conformation. This technique, however, would be unsuitable for determination of subunit size estimation of aggregated proteins as these proteins are held together in a complex, which must be destroyed by denaturing agents such as detergents and reducing agents prior to M_r estimation.

The previous results have suggested that the ß-lactamases of pI<6.8 all share similar, if not, identical M_r. The rationale is, therefore, that native PAGE would be unable to separate these enzymes by their M_r but could separate them by their charge. The enzymes would also be arranged in their native state, with their biological activity intact and could be detected with nitrocephin.

7.9.1. Methodology

The separations were run on a Phastgel Homogeneous 12.5 mini-gel (Pharmacia). The buffer system in the Native gel buffer strips was 0.88 M L-alanine and 0.25 M Tris, pH 8.8. The buffer strips were made of 2% agarose IEF.

The fractions from the previous anion exchange step that showed greatest ß-lactamase activity (6, 11, 12) were concentrated to 200µl in a Centricon® concentrator prior to separation. These fractions and the partially purified enzyme preparation that was originally applied to the Mono Q column were applied to the gel. Four µl of each enzyme preparation was loaded at the cathode. The gel was run as recommended by the manufacturer. ß-lactamase activity was visualised by emersing the gel in nitrocephin solution (500mg/l) for up to 5 minutes.

7.9.2. Results

The result of the Native PAGE gel is shown in figure 31. The original sample that had not been passed through the anion exchange column revealed the presence of four distinct bands of ß-lactamase activity, compatible with the concept of four ß-lactamases (Lane 1). After prolonged staining, each sample that had previously been through the anion exchange step (fractions 6, 11 and 12) (lanes 2-4) revealed the presence of 2-3 ß-lactamase bands, although this is not clear in the figure. These fractions (Lanes 2-4) stained weakly but did demonstrate that the enzymes with the lower isoelectric point migrated to the anode at a faster rate than the enzymes of lower pI. It might be assumed that the enzyme of pI 6.8 was responsible for the ß-lactamase activity closer to the cathode, not only because of its lower density of negative charge but also because of its high M_r, and the other three bands closer to the anode portion of the gel were the ß-lactamases of pI<6.8.

7.9.3. Conclusions

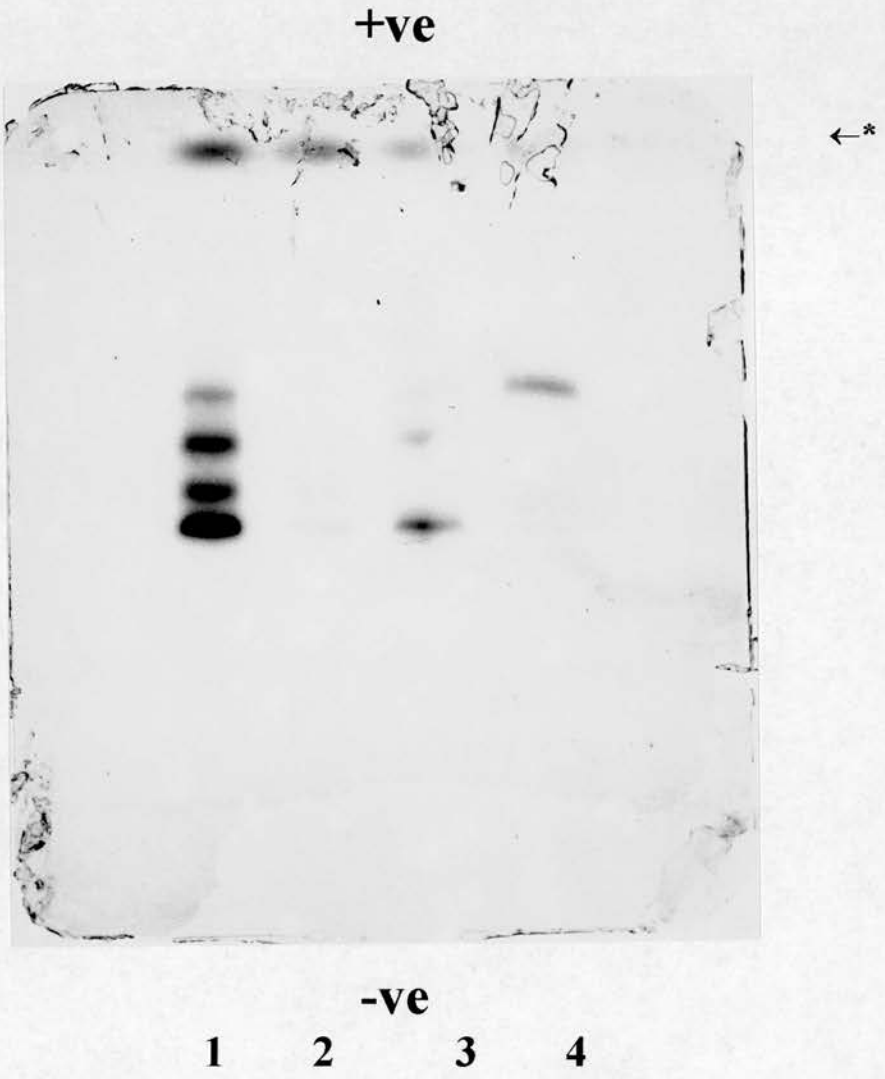
Although electrophoresis, under nondenaturing conditions, can provide useful information concerning the physical characteristics of proteins, it cannot be assumed that the proteins are all in the shape of linear molecules and in this instance is probably unsuitable for M_r calculations, as it is thought that these enzymes are aggregated into sub-unit conformation (it should be noted that Ferguson plot analysis may be used for M_r calculations, although this method involves running the unknown and several standards in at least five gels of different total concentrations, it is not suitable for M_r estimations of proteins in subunit form [237].).

Native PAGE has provided no new information on the M_r of these enzymes, it **has** complemented the results obtained with the electrodialysis experiment and provided additional proof for the concept of four discrete ß-lactamases. Moreover, it has also shown that the ß-lactamases present have not been purified adequately or in sufficient quantities to allow further studies on each discrete enzyme. Clearly, with the techniques available, these separate ß-lactamases were impossible to purify to homogeneity. However, the ß-lactamases could be separated by gel filtration into two fractions, a ß-lactamase with a pI of 6.8, and the other fractions containing the bands of lower pI (pI<6.8).

These fractions were used for further study.

Figure 31.

Native PAGE by the PhastSystem employing a homogeneous 12.5 mini-gel on *X. maltophilia* 5B105 ß-lactamases previously purified by anion exchange chromatography



Four µl of ß-lactamase preparation loaded at cathode

*Lane marker dye

Lane 1: concentrated ß-lactamase preparation from *X. maltophilia* 5B105

Lane 2: fraction 6 from anion exchange step

Lane 3: fraction 11 from anion exchange step

Lane 4: fraction 12 from anion exchange step

The determination of the presence of sub-units involves characterisation of the protein under conditions which favour association, followed by analysis under conditions which favour dissociation. One simple approach is to carry out electrophoresis under nondenaturing conditions (this we have already accomplished, with the use of low pressure gel filtration), followed by a second dimension under denaturing conditions.

Sodium dodecyl-polyacrylamide electrophoresis (SDS-PAGE) is one of the most common electrophoresis methods used today and is an ideal method to examine these enzymes under denaturing conditions. In practice, the proteins are first dissociated into their polypeptide subunits by hot SDS, the mixture binds tightly to the protein imparting a net negative charge, concomitant treatment with ß-mercaptoethanol (a reducing agent which disrupts disulphide bonds) breaks proteins down to their constituent sub-units. This technique would allow us to determine not only the M_r of the subunits (if any), but also their conformation.

It was decided to employ SDS-PAGE to calculate the subunit size of these ß-lactamases.

7.10. SDS-PAGE on the PhastSystem®

7.10.1. Methodology

Sample preparation

Equal amounts of the concentrated ß-lactamase extracts obtained from the G-150 gel filtration column containing either the ß-lactamase of pI 6.8 or the enzymes of pI <6.8 were treated with 5.0% ß-mercaptoethanol and 2.5% SDS at 100°C for 5 minutes [238]. Four µl of sample was applied to a Phastgel gradient 10-15 mini-gel, and subjected to electrophoreses as per manufacturer's instructions.

This thesis has shown that purification of the ß-lactamases to homogeneity has been unsuccessful, and the fractions obtained by gel filtration contain other contaminating proteins that would also stain by conventional protein staining methods, such as Coomassie brilliant blue and silver nitrate staining.

A technique was required that would permit the biological activity of the ß-lactamases to re-establish, and hence allow subsequent staining by nitrocephin.

7.10.2. Renaturation of ß-lactamase activity

Massidda *et al.* [140], demonstrated that the *Cpha* ß-lactamase from *Aeromonas hydrophila* could be renatured after SDS-PAGE, and the biological activity of the enzyme regained.

7.10.3. Methodology

The technique involved incubating the gel in 50 mM Tris Cl buffer (pH 8.0) containing 1% Triton X-100 and 0.1 mM zinc sulphate for 4 hours at 37^{o}C in a petri dish. Triton-X is a non-ionic detergent, which removes the bound SDS, the sulfhydryl groups of the enzyme are slowly oxidised. The protein then refolds to the native enzyme and recovers its enzymatic activity. The zinc sulphate was included to enhance any metallo-ß-lactamase activity.

7.10.4. Staining

After renaturation was complete, visualisation of ß-lactamase activity was obtained by staining with nitrocephin as before. Low molecular weight protein standards (Biorad) were run simultaneously under the same conditions and stained with Coomassie brilliant blue R250. The molecular weight standards were transposed over the gel with the renatured ß-lactamases to enable M_r to be ascertained.

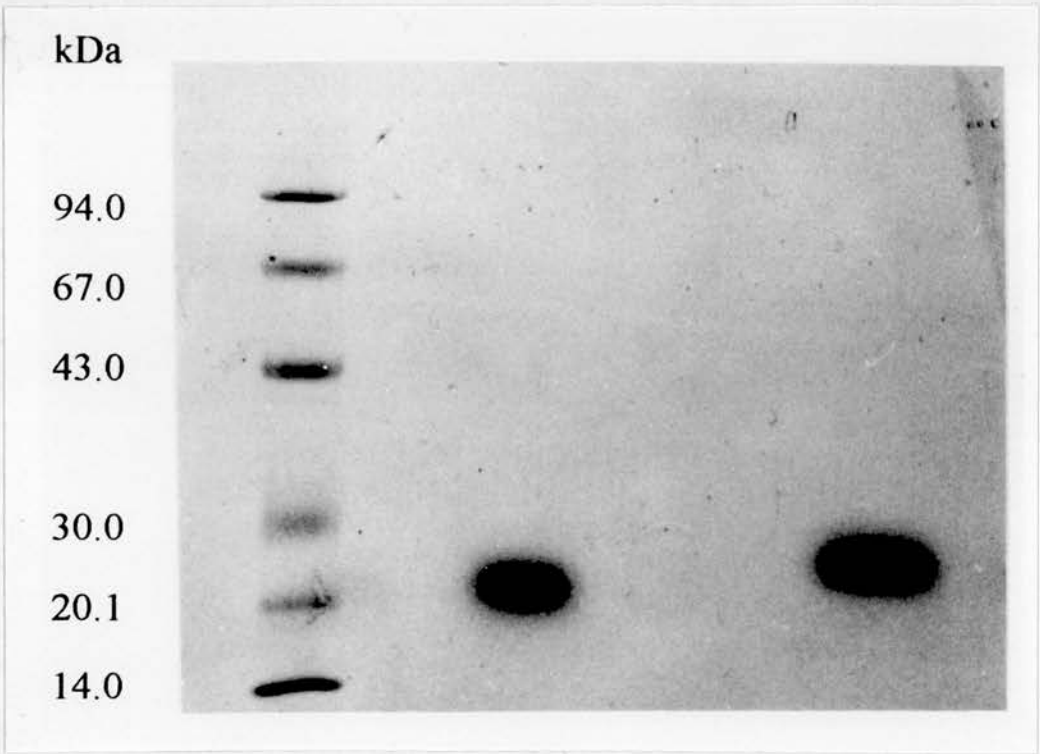
7.10.5. Results

SDS-Page analysis followed by gel renaturation treatment (figure 32) showed in each case, the presence of a single band of ß-lactamase activity, with an apparent M_r of 26 kDa for the enzyme of pI 6.8, whilst the other ß-lactamase bands showed the presence of a single protein with an apparent M_r of 24 kDa.

The data suggest that the conformation of the enzyme of pI 6.8 is a tetramer of four subunits which are very similar in M_r. The other ß-lactamases between them exhibited a single band of ß-lactamase activity on SDS-PAGE, indicating dimeric enzymes comprised of two subunits of similar size.

Figure 32.

SDS-PAGE by the PhastSystem of the ß-lactamases from *X. maltophilia* 5B105.



Lane 1: molecular weight standards

Lane 2: ß-lactamase bands of pI< 6.8

Lane 3: ß-lactamase band of pI 6.8

7.11. Hydrolysis of ß-lactam antibiotics

In preliminary experiments, the ß-lactamase of pI 6.8 was found to be unstable in 50 mM sodium phosphate buffer (pH 7.0) at 37°C and 80% of the activity was lost after 5 minutes incubation in the buffer. The enzyme was stable in 50 mM Tris Cl with an optimum pH of 8.0. Therefore all assays on the ß-lactamase of pI 6.8 were performed with this buffer. The other ß-lactamases were stable in phosphate buffer, assays for these enzymes were performed with 50 mM sodium phosphate buffer (pH 7.0).

Assays of ß-lactamase hydrolysis of various ß-lactams were performed with the partially purified extracts from the G-150 column on the ß-lactamase of pI 6.8, and the ß-lactamases of lower pI (<6.8) For convenience, these ß-lactamases shall be designated XM-A, and XM-B respectively. The Michaelis constant (K_m) and the maximum rate of hydrolysis (V_{max}) were determined by assaying the enzyme in decreasing substrate concentrations. The results are presented in table 46. The values are expressed as a percentage of the value for penicillin.

XM-A was primarily a penicillinase and also readily hydrolysed imipenem and meropenem, it was much less active against all classes of cephalosporins, although the affinity (low K_m) for cephalosporins was higher than that for penicillins and imipenem. Aztreonam was not hydrolysed. Between them the XM-B enzymes exhibited a broad substrate profile, hydrolysing an extensive range of ß-lactams but not imipenem, meropenem, ceftazidime or aztreonam. Affinity for cephalosporins was higher than for penicillins.

Table 46.

Hydrolysis of ß-lactam antibiotics by XM-A and XM-B ß-lactamases from *X. maltophilia* 5B105

Substrate	XM-A			XM-B		
	V_{max}[a]	Relative[b] V_{max}	K_m (mM)	V_{max}[a]	Relative[b] V_{max}	K_m (mM)
Penicillin	14.3	100	0.6	1.2	100	0.108
Ampicillin	5.71	39.9	0.34	2.3	191.6	0.22
Carbenicillin	2.0	13.9	0.67	0.45	37.5	0.55
Azlocillin	5.5	38.4	0.24	1.25	104	0.22
Cephaloridine	0.09	0.6	0.22	1.8	150	0.05
Cefuroxime	0.66	4.6	0.133	0.28	23.3	0.076
Cefotaxime	0.303	2.1	0.05	0.19	15.8	0.08
Ceftazidime	0.03	0.2	0.09	NMH	-	-
Meropenem	1.29	9.0	0.057	NMH		
Imipenem	3.33	23.3	0.25	NMH	-	-
Aztreonam	NMH	-	-	NMH	-	-

[a] µmols of substrate hydrolysed/min/ml of enzyme solution.

[b] Relative to penicillin 100%.

NMH , No measurable hydrolysis.

7.12. Inhibitor studies

Table 47 shows the effect of the ß-lactamase inhibitors, potassium clavulanate and BRL 42715, expressed as the concentration required for 50% inhibition (ID_{50}) of enzymatic activity. No inhibition of the enzymatic activity of the XM-A enzyme was observed by either inhibitor. The XM-B enzymes were sensitive to both potassium clavulanate (ID_{50} = 0.16 µM) and BRL 42715 (ID_{50} = <0.001 µM).

Table 47.

ID_{50} values (µM) of potassium clavulanate and BRL 42715

Inhibitor	XM-A[1]	XM-B[2]
Potassium clavulanate	>50	0.16
BRL 42715	>10	<0.001

[1] Imipenem (100 µM) used as test substrate.

[2] Cephaloridine (100 µM) used as test substrate.

Table 48 shows the effect of 0.1 mM EDTA, 0.5 mM mercuric chloride, 0.5 mM p-CMB.

Table 48.

Effect of various inhibitors on the activity of the XM-A and XM-B ß-lactamases.

Inhibitor	Conc[n]. (mM)	% Inhibition of:	
		XM-A[1]	XM-B[2]
p-chloromercuribenzoate	0.5	69	100
Mercuric chloride	0.5	70	100
EDTA	0.1	100	0

[1] Nitrocephin (100 µM) used as test substrate.

[2] Cephaloridine (100 µM) used as test substrate.

Treatment of XM-A with EDTA resulted in complete inhibition of enzymatic activity. The inhibition was completely reversed after dialysis against 50 mM Tris Cl (pH 8.0) for 30 minutes at 37°C, and addition of 0.1 mM zinc sulphate to the reaction mixture. Enzymatic activity was partially inhibited by mercuric chloride and p-CMB. The XM-B enzymes were not affected by the addition of EDTA, but were completely inhibited by the addition of both p-CMB and mercuric chloride.

7.13 Conclusions

We have shown that the XM-A enzyme is a metallo-ß-lactamase and clearly belongs in group 3 (metallo-ß-lactamases (MET-N) in the classification scheme of Bush [149]. The XM-B group of enzymes appear to be well placed in Bush Class 2b' (extended broad spectrum ß-lactamases inhibited by clavulanic acid (EBS-Y) [172]; however, without separation of each individual ß-lactamase, it is impossible to say with certainty, if they share similar biochemical properties.

The similarities of the physical and biochemical characteristics of the XM-B ß-lactamases to the ubiquitous TEM plasmid-mediated ß-lactamases did not go unnoticed.

The following should be noted: The XM-B enzymes share similar isoelectric points, subunit M_r, substrate profile. They are also inhibited by potassium clavulanate. The hypothesis that the XM-B group of ß-lactamases may have an ancestral relationship with the TEM group was investigated.

7.14. PCR-amplification of genomic DNA from *X. maltophilia* 5B105

A simple technique to study this hypothesis was to perform the Polymerase Chain Reaction (PCR) on genomic DNA isolated from *X. maltophilia* 5B105 with universal TEM oligonucleotides (*Bla*3' and *Bla*4'). Any target TEM DNA present would serve as a template for amplification of the TEM gene. The sequences of the oligonucleotides used are shown in figure 33.

Figure 33.

The sequences of the oligonucleotides, *Bla*3' and *Bla*4'

Bla3':

5'-CTC TCT AGA AAA AGG AAG AGT ATG AGT ATT-3'

Bla4':

5'-CTC GCA TGC GTA AAC TTG GTC TGA CAG TTA-3'

PCR was performed on total genomic DNA preparations of *X. maltophilia*. *E. coli* harbouring plasmids encoding for either TEM-1_{R1} or SHV-$1_{R1010-6}$ production were included as controls. Ten percent of the reaction mixture was applied to a 1% agarose gel. After electrophoresis, the gel was stained with ethidium bromide solution and visualised under UV transillumination.

7.14.1. Results

The result of PCR on genomic DNA from *X. maltophilia* 5B105 is shown in figure 34. Amplification of any structural TEM DNA occurred only with the TEM control. No amplification of TEM DNA was observed from either the DNA of *X. maltophilia* 5B105 or the *E.coli* encoding for SHV-1 ß-lactamase.

7.14.2. Conclusions

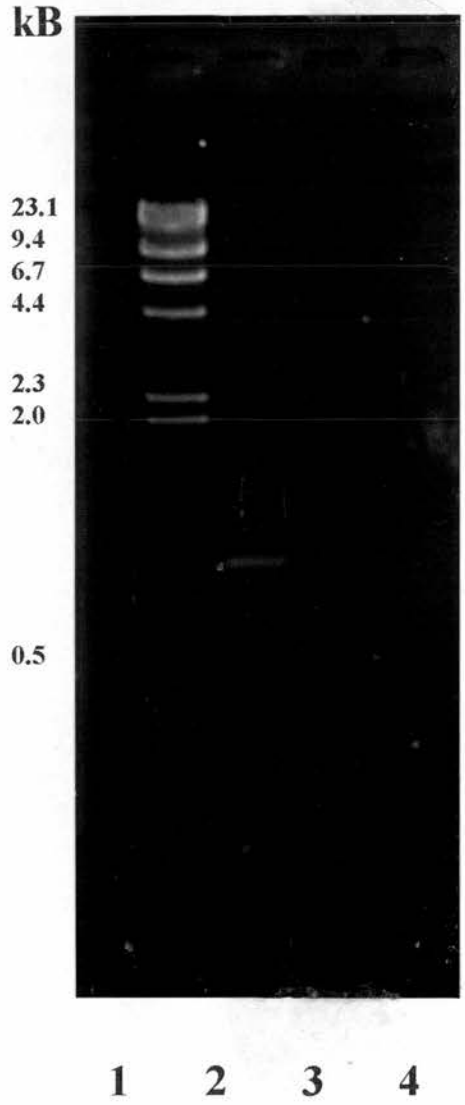
This result demonstrates that the there is no significant homology within the chromosomal DNA of *X. maltophilia* 5B105 to the TEM structural gene.

8.0. Utilisation of selective pressure on *E. coli* with imipenem

It was originally thought unlikely that the common plasmid-mediated ß-lactamase genes, in particularly the TEM-1 gene would mutate to encode for ß-lactamases with the capability to hydrolyse third generation cephalosporins or show increased resistance to ß-lactamase inhibitors. We now know that this early assumption was misplaced with numerous reports of ESBLs in the literature. Payne [235] and Thomson & Amyes [239], demonstrated that the application of selective pressure with either third generation cephalosporins or co-amoxiclav *in vitro*, on

Figure 34.

Agarose gel electrophoresis of PCR amplified products from genomic DNA of *X. maltophilia* 5B105



Lane 1: λ DNA digested with *hin*d III

Lane 2: genomic DNA preparation from *E. coli* $R1_{TEM-1}$

Lane 3: genomic DNA preparation from *E. coli* $R1010\text{-}6_{SHV-1}$

Lane 4: genomic DNA preparation from *X. maltophilia* 5B105

E.coli harbouring plasmids encoding for TEM-1 ß-lactamase resulted in selection of ESBLs or TEM enzymes resistant to inhibitors (TRC-1) respectively. The same assumption has been made for the carbapenems, although in this case, the assumption may be correct. To date, there has been no reports of TEM or SHV derived ß-lactamases with increased resistance to carbapenems. The object of this experiment was to determine if *E. coli* expressing TEM-1 ß-lactamase could be mutated *in vitro* to carbapenem resistance.

8.1. Methodology

The method employed was based on that described by Thomson & Amyes [239]. The antibiotic susceptibility of the host organism expressing the TEM-1 ß-lactamase, *E. coli* J62-2_{R1} to imipenem (0.12 mg/l) was determined. Twenty µl of an overnight culture of *E. coli* J62-2_{R1}, encoding for the TEM-1 ß-lactamase in IST broth was inoculated into three 100ml volumes of IST broth containing 0.12 mg/l imipenem, 0.25 mg/l imipenem and 0.5 mg/l imipenem respectively. After overnight incubation at 37°C with constant shaking, the cultures that had grown were subcultured into the same broth/antibiotic mixture. This procedure was repeated over five days. After the final subculture, 10 ml of broth from each flask that exhibited growth was removed. The bacteria were harvested by centrifugation (2500 x *g*., 15 min). The bacterial pellet was resuspended in the same volume of single strength Davis-Mingioli minimal media (SSDM). This was further diluted 10^{-6} in SSDM and 0.1 ml of dilution spread onto IST agar containing imipenem at the same concentration as the strain had been cultured in.

8.2. Results

The flasks containing 0.5 mg/l imipenem remained clear throughout. Five single colonies were selected from the plates that contained either 0.12 mg/l or 0.25 mg/l imipenem. The auxotrophic requirements of the colonies selected were examined to ensure they were *E. coli* J62-2.

8.2.1. Antibiotic susceptibilities

In total, 10 colonies of *E. coli* J62-2_{R1} were assessed for imipenem resistance. MICs for five colonies challenged with 0.12 mg/l imipenem were identical to those of the

original host (0.12 mg/l). The five colonies challenged with 0.25 mg/l imipenem all displayed MIC levels of 0.25 mg/l to this drug. Stoke's sensitivity testing on all colonies, employing the original *E. coli* J62-2 as control, showed no reduction in zone size to any other ß-lactam.

8.2.2. Isoelectric focusing

The 10 colonies selected were used to prepare small scale ß-lactamase preparations. These were subjected to isoelectric focusing employing ampholines with a pH range of 3.5-10.6. All colonies co-focused at exactly the same pI as TEM-1 ß-lactamase from the original *E. coli* strain (data not shown).

8.3. Conclusions

Despite a single dilution increase in resistance to imipenem by five colonies of *E. coli* J62-2_{R1} there was no alteration in enzyme production, with the TEM-1 ß-lactamase produced on each occasion. Although not examined here, the slight increase in MIC to imipenem in these strains is unlikely to be associated with increased levels of enzyme production as no concomitant resistance to other ß-lactams was observed. The most likely explanation for this increase may be as a result of permeability changes or altered PBPs, although this has not been examined in this thesis.

We have shown in our experiments that the TEM-1 gene appears to be unable to undergo mutation to allow hydrolysis of imipenem. This was perhaps anticipated. Despite the steady global increase in the use of imipenem, especially in those areas where treatment with third generation ß-lactams has failed, there have been no reports of TEM-derived ß-lactamases conferring resistance to carbapenems; at least for the moment.

CHAPTER 4 DISCUSSION

1.0. Prelude

The initial aim of this thesis was to examine the antibiotic resistance levels of Gram negative aerobic bacilli responsible for septicaemia over a 12 year study period. There have been reports concerning the development of resistance by these organisms over the past decade, to all classes of ß-lactam antibiotics. This resistance has prompted the search for newer, more effective antibiotics and has perhaps culminated in the discovery, and use, of the most powerful ß-lactam family to date; the carbapenems. As previously discussed, resistance to these drugs by virtue of OMP changes has been well documented but carbapenem resistance mediated by metallo-ß-lactamases has been confined to relatively non-pathogenic species. At the concept of this thesis, no serine ß-lactamase on its own, had been shown to mediate resistance to this group of antimicrobials.

The blood culture stock collection from the clinical bacteriology section of the Dept. of Medical Microbiology, Edinburgh University, has afforded a unique opportunity to examine the *in vitro* susceptibility of a range of organisms responsible for serious sepsis. This collection represents strains collected prior to the introduction of the second and third generation cephalosporins and latterly the introduction of carbapenems and quinolones, into clinical practice. Subsequently, over the years, these strains have endured a progressive increase in the use of more powerful and broader-spectrum antibiotics, and hence the opportunity to develop resistance by selection.

In this thesis, the incidence of resistance to various antimicrobials within the population of Gram negative aerobic bacilli has been investigated. Strains resistant to cefuroxime have been studied, to detect any evidence for the evolution of extended spectrum TEM and SHV ß-lactamases in our hospital. Carbapenem resistance has been examined, culminating with the discovery of the ß-lactamase, ARI-1, in a strain of *A. baumannii*, the first documented serine active site carbapenemase.

The species *X. maltophilia* is the only Gram negative aerobic bacillus isolated with any frequency from clinical material resistant to imipenem. The ß-lactamase complement of this species has been examined and clarified.

These results and their significance are discussed in the following chapter.

2.0. The prevalence of Gram negative aerobic bacilli collected over a 12 year period

Gram negative bacilli (1453 strains) were collected over a 12 year study period. As expected *E. coli* was the predominant isolate (53%) with *Klebsiella* spp. and *Pseudomonas* spp. the next most prevalent (10%). These results are comparable with a three year UK survey performed by Spencer *et al.* [240] who reported the frequency of hospital isolates that were *E. coli* as 57% with *Pseudomonas* spp. and *Klebsiella* spp. as 9% and 5% respectively. Although it should be noted that these figures represent strains isolated from a general survey rather than from a specific site.

In parallel with the overall incidence of isolation, *E. coli* remained the most frequently isolated pathogen throughout the entire study period. There appeared to be no obvious trend to the regularity of isolation of any particular species, although Phillips *et al.* [241] reported an overall 4% to 6% increase in the isolation of those organisms that produce a Class I cephalosporinase over a 20 year period in their study.

2.1. Resistance of the survey strains to various antimicrobials

The highest incidence of resistance to all strains was for ampicillin (63%). Resistance to aminoglycosides was surprisingly high (gentamicin 12%, tobramycin 15% and netilmicin 21%), although cumulative MICs indicated that most of these resistant strains fell close to the recommended breakpoint value (1mg/l). In a similar study, Phillips *et al.* [241] employed an intermediate breakpoint value of 4 mg/l for aminoglycosides. A similar value employed in this study, would decrease resistance levels to approximately 10% or less for all species against all aminoglycosides tested. Aminoglycoside serum levels of >1 mg/l are readily achievable, and the general application of a breakpoint value of 4 mg/l may be more relevant to the clinical setting.

Ceftazidime was the most effective cephalosporin with 16% resistance, cefotaxime resistance was 21% and cefuroxime 32%. The most effective agents were the carbapenems, imipenem and meropenem with resistance levels of 4% and 2% respectively. The only quinolone tested, ciprofloxacin, demonstrated an efficacy commensurate with that of the carbapenems (3%).

When the resistance pattern of individual species was analysed the results were much as expected. The *Pseudomonas* spp. and *X. maltophilia* displayed the most impressive pattern of resistance to almost all antimicrobials. The Enterobacteriaceae were exquisitely sensitive to the carbapenems with the exception of the *Proteus* spp. Ten per cent of all strains were resistant to imipenem while meropenem was more active with only 2.8% resistance. These results are in broad agreement with those reported by Edwards and Turner [242], who demonstrated in a European multi-centre survey that approximately 20% of 1280 strains of *Proteeae* were resistant to imipenem but sensitive to meropenem. It is known that imipenem is intrinsically less active than meropenem [242], this resistance is thought to be as a result of OMP changes [54,56], rather than ß-lactamase mediated.

The species known to produce a Class I cephalosporinase were generally more resistant to antimicrobials particularly to the cephalosporins than those organisms such as *E. coli* and *Klebsiella* spp. that do not produce these enzymes. Only a strain of *A. baumannii* was resistant to both carbapenems.

These results have demonstrated the excellent *in vitro* efficacy of carbapenems and ciprofloxacin against those species that cause Gram negative sepsis, and the use of these drugs may be worthwhile in this setting. Other workers [243] have recommended the use of carbapenems for empirical therapy for both Gram positive and Gram negative sepsis without the administration of a codrug such as an aminoglycoside.

The importance of such susceptibility data cannot be underestimated. Spencer [240] made the following points concerning susceptibility data:

1. Locally, surveillance can improve the quality of antibiotic prescribing for the individual and influence the antibiotic usage in hospital.

2. Nationally, it can assist the formulation of policies for the supply and use of antibiotics in man or animals and encourage responsible action by antibiotic manufacturers in the promotion of their products.

3. The results would supply clinicians with the latest surveys of the prevalence of pathogenic micro-organisms and their susceptibility patterns.

4. The information gathered may be used to monitor the incidence of resistance and changes in the prevalence of resistance to newly released antibiotics.

2.2. Cefuroxime-resistant strains

Two hundred and forty five cefuroxime resistant strains (MIC>4 mg/l) were selected from the original survey strains for further study. This group excluded those species such as *X. maltophilia* and *Pseudomonas* spp. that are intrinsically resistant to this compound.

The species represented within this sub-group were very much as expected with the majority of these organisms known to produce a Class I cephalosporinase (61% *Enterobacter* spp., 59% *Acinetobacter* spp., 37% *Serratia* spp., 12% *Citrobacter* spp. and 15% *Proteus/Morganella/Providencia* spp).

Almost all of the cefuroxime resistant strains were concomitantly resistant to ampicillin (93%), and cross resistant to most other classes of antimicrobials. The carbapenems were the most efficacious (<1.0% resistance).

2.3. ß-lactamase production by the cefuroxime-resistant strains

Of the 245 cefuroxime-resistant strains examined; 198 produced a chromosomal enzyme alone, 28 produced a chromosomal enzyme plus the TEM-1 ß-lactamase, 1 produced a chromosomal ß-lactamase plus the SHV-1 ß-lactamase, 6 produced the TEM-1 ß-lactamase alone. None of the strains demonstrated the presence of transferable ESBLs.

These findings suggest that the cefuroxime resistance in most of these strains is mediated by "typical" Class I chromosomal cephalosporinases, and it appears that these enzymes are more problematic from the clinical aspect than the ESBLs. Although it should be borne in mind that other mechanisms such as permeability changes and affinity for PBPs should not be discounted. The ESBLs that have been reported in several other countries, at present, do not appear to be a problem in our

hospital, despite a steady increase in the use of second and third generation cephalosporins. However, these results should not let us become complacent. In Glasgow Royal Infirmary it appears that ESBLs, similar to the SHV-derived ESBLs may be present (Dr C. Thomson, personal communication), and continued vigilance, with respect to screening programmes should be a priority.

These results are similar to those obtained by Hood [13]. Hood examined 200 general isolates of cefuroxime-resistant Gram negative bacilli from the same hospital, and found no evidence of transferable ESBLs.

It is not thought that the increased use of imipenem over the last few years should become a major concern. The carbapenems have no potential for selecting the constitutively derepressed subpopulation, neither does the induced high level enzyme production affect the efficacy of the antibiotic, furthermore, they are they not thought to select ESBLs derived from TEM or SHV ß-lactamases. However, the discovery of novel ß-lactamases is a more difficult problem to address.

3.0. Discovery of ARI-1 (*Acinetobacter* resistant to imipenem), a carbapenemase, in *A. baumannii* 6B92

As previously alluded, the only isolate to display resistance to the carbapenems among the cefuroxime-resistant population was a strain of *A. baumannii* 6B92 with MICs to imipenem and meropenem of 16.0 mg/l and 32.0 mg/l respectively. The isolate was also resistant to all other ß-lactams tested, but not gentamicin or ciprofloxacin.

Although carbapenem resistance in *Acinetobacter* spp. has been reported [60,188], this resistance has been attributed to changes in affinity for PBPs [60] or the interplay between penicillin binding proteins with altered expression and/or affinity and decreased permeability of the outer membrane [184], as a consequence of treatment with carbapenems. To our knowledge, there have been no reports of enzymatic degradation of carbapenems by ß-lactamases in this species.

This strain was isolated in 1985 from a surgical ward in Edinburgh Royal Infirmary, long before the introduction of carbapenems in this hospital in early 1990. Although the medical records of this patient have not been studied, it was unlikely that this

resistance had been selected by the use of any other antimicrobial. As already discussed, ESBLs or hyperproduction of Class I ß-lactamases on their own have no significant hydrolytic action on carbapenems without a concomitant increase in impermeability, and it has already been suggested that carbapenems enter the cell by a different route from other agents [58]. Therefore any selective pressure by other ß-lactams would be highly unlikely to select carbapenem resistance. The sensitivity of this organism to ciprofloxacin and gentamicin would also intimate that the carbapenem resistance was ß-lactamase-mediated, and not a permeability mutation.

3.1. Isoelectric focusing

A ß-lactamase of pI 6.65 was identified in this isolate, as well as a typical chromosomal Acinetobacter cephalosporinase of pI >9.0. The isoelectric point of 6.65 is similar to those values exhibited by plasmid-mediated enzymes, rather than a chromosomal enzyme. These chromosomal enzymes are usually of basic pI. Plasmid-mediated ß-lactamases do exist in *Acinetobacter* spp. but they have usually been penicillinases, which have previously been identified in *Pseudomonas* spp. or the Enterobacteriaceae [244]. The same workers did report a carbenicillinase of pI 6.3 in several strains of *Acinetobacter* spp., designated CARB-5, however none of these strains exhibited carbapenem resistance.

3.2. Molecular mass

The ARI-1 ß-lactamase demonstrated a M_r of 23kDa by gel permeation, the other enzyme identified exhibited a M_r of 58kDa. The M_r of the ARI-1 enzyme is clearly different from the M_r of those enzymes normally produced by the species. Hood demonstrated that the M_r of these enzymes is usually >60kDa [229].

3.3. Substrate profile

Hydrolysis of ß-lactam substrates by ARI-1 was very slow and only hydrolysis of penicillin, ampicillin, azlocillin and cephaloridine could be demonstrated by spectrophotometric means. The substrate profile **did** indicate that ARI-1 was a penicillinase rather than a cephalosporinase, most other carbapenemases have also been shown to hydrolyse penicillins preferentially [128], this is not surprising considering both classes of ß-lactams have a 5-membered ring.

The microbiological assay however, clearly demonstrated that the enzyme possessed the ability to inactivate imipenem and azlocillin but not any of the second or third generation cephalosporins. The inability of the spectrophotometric assay to demonstrate direct hydrolysis of carbapenems was puzzling, however, it is known that this technique is not suitable for assay of all ß-lactamases, especially if that hydrolysis is extremely slow. Microbiological assay is extremely sensitive and although not as convenient, a very useful technique to demonstrate the hydrolytic action of these ß-lactamases.

It has been postulated, particularly for inducible ß-lactamases, that the ß-lactamase protects the cell by "binding" rather than hydrolysing the antibiotic molecules as a result of the low K_m of these enzymes. It has been said that these ß-lactamases act as "periplasmic sponges" that prevent antibiotics from reaching their target sites on the cytoplasmic membrane [245,246]. Alternately, Vu and Nikaido [247] have proposed that the contribution of trapping alone to the resistance level is of negligible magnitude in comparison with the contribution of hydrolysis, even in species with fairly low outer membrane permeability. They calculated that trapping alone would raise the MIC by only around 2mg/l whereas slow hydrolysis would explain a much higher increase. They state that the periplasmic concentrations of ß-lactamase are much higher than those observed in *in vitro* assays, and secondly, enzyme-antibiotic affinity ensures that the drug turnover remains efficient, even at low substrate concentrations.

It seems most likely therefore, that the inactivation of imipenem by ARI-1 is probably as a result of extremely slow hydrolysis in concert with high affinity for the substrate.

3.4. Inhibitor studies

Although the ARI-1 ß-lactamase demonstrated physical properties similar to those of the broad-spectrum plasmid-mediated ß-lactamases such as TEM and SHV, unlike these enzymes it was not inhibited by the action of potassium clavulanate. It was, however, inhibited by BRL 42715 a mechanism-based ß-lactamase inhibitor, which inactivates serine ß-lactamases but not those with a metal ion at their active site. Coupled with the non-inhibition by EDTA these results strongly suggest that the ARI-1 enzyme is a serine active site ß-lactamase rather than a zinc centred enzyme, and as such, is the first reported isolation of a serine active site enzyme with significant

activity against carbapenems. ARI-1 was also very susceptible to the carbapenems, meropenem and imipenem when used as inhibitors, much more so than TEM-1. This shows that the enzyme binds these substrates and is indicative of the high affinity of ARI-1 for carbapenems, and is further evidence for the binding/slow hydrolysis hypothesis discussed in the preceding section.

3.5. Genetic studies

The previous results demonstrating the clear dissimilarity of the ARI-1 ß-lactamase from the normal complement of Acinetobacter ß-lactamases, infers that the gene encoding the enzyme was introduced from an extraneous source; yet, despite extensive experimentation to demonstrate the presence of, or transfer of an extrachromosomal source, no resistance plasmid could be visualised or transferred. The existence of a small plasmid of 6.5kb was observed, however, it would be unlikely that antibiotic resistance genes would be carried on such indigenous plasmids, and in any event, probably not carry the necessary information for genetic transfer to occur [248]. It is known that genetic studies on *Acinetobacter* spp. are notoriously difficult to perform.

Unlike some species, such as *Aeromonas* spp., and *X. maltophilia* which appear to have a paucity of plasmids, plasmids **are** common amongst strains of Acinetobacter. Gerner-Smidt [222] demonstrated the existence of indigenous plasmids in 75 of 93 strains of *Acinetobacter* spp. examined, with a range of 0-20 plasmids/strain. The majority were less than 23 kb in size, with two thirds of strains harbouring two or more plasmids.

The more likely explanation for the failure to detect an extrachromosomal source is that the resistance gene is carried on a large plasmid that has broken up during the extraction process, and hence we have failed to isolate it, despite the utilisation of a variety of plasmid extraction procedures. This plasmid, despite it's presumed large size, was not transferable. Lambert *et al.* [249] stated that the failure to transfer resistance genes between *Acinetobacter* spp. and other organisms did not result from lack of expression of Acinetobacter genes in heterologous systems, but rather from defects in conjugation donor or recipient ability, or plasmid replication in the new host, or both.

Plasmid transfer from *Acinetobacter* spp. to other genera, has been shown to be difficult. In other studies it has been shown that self transmissible R plasmids transferred from *E. coli* to *Acinetobacter* spp. required a mobilising plasmid for re-transfer to occur [250]. In our experiments the use of the *inc*P group plasmid, RP4, failed to mobilise any resistance gene. RP4 was chosen because it, and its relatives, have been known to mobilise the chromosomes of a variety of Gram negative bacteria, although at a low frequency [251].

It may have been useful to attempt transfer of any resistance gene on solid surfaces, as Towner and Vivian [252] demonstrated that transfer only occurred at detectable frequency on solid surfaces, not in liquid mating. Moreover, Towner [248] suggested that tests with a single "representative" plasmid, even those of the same incompatibility group, cannot always be regarded as conclusive as a result of their divergent behaviour in the host system, and it may have been prudent to attempt mobilisation of the ARI-1 gene with a different plasmid.

In conclusion, it is most probable that the failure to detect plasmid-mediated transfer of an antibiotic resistance gene for ARI-1 may simply reflect the absence of a suitable test system rather than the absence of a plasmid. Another possible scenario is the resistance gene **has** originally been plasmid mediated but has been unstable in the host system and incapable of sustained stable maintenance, and subsequently undergone introduction into the host chromosome by transposition. There is much evidence for this hypothesis in *Acinetobacter* spp. Bergogne Bérézin and Joly-Guillou [188] stated that much of the resistance mechanisms observed in *Acinetobacter* spp. were essentially identical to those encoded by genes carried on plasmids commonly found in the Enterobacteriaceae.

3.6. Plasmid curing experiments

Despite the failure to isolate or visualise a resistance plasmid, the presence of a plasmid location for the ARI-1 gene was strengthened by the confirmation that the production of the enzyme can be cured by the addition of ethidium bromide to the growth medium. The rate of cure was 10%, this is in contrast with previous work by Goldstein *et al.* [253] who, by employing the same plasmid curing technique with *A. calcoaceticus* BM200 demonstrated a rate of cure of resistance traits of only 0.3% (1 of 300 colonies). The rate of cure of resistance plasmids in Enterobacteriaceae appears higher than that in Acinetobacter. Bouanchaud *et al.* [232] employing

ethidium bromide, reported loss of resistance traits at high frequency in *E. coli* K12, ranging from 30% to 70%. Our results, as well as those of Goldstein, appear to suggest that resistance plasmids present in *Acinetobacter* spp. are not as easily eliminated by ethidium bromide curing as those found in Enterobacteriaceae.

The loss of the ARI-1 ß-lactamase coincided with the concurrent loss of high levels of resistance to penicillins and all resistance to carbapenems, whose MICs fell to basal levels normal for the species. The residual resistance to the penicillins is probably as a result of some hydrolysis by the cephalosporinase of pI >9.0. Carbapenems are resistant to the hydrolytic action of Acinetobacter ß-lactamases.

3.7. The action of BRL 42715 on the MIC values to carbapenems

Despite the evidence of the presence of a novel ß-lactamase in *A. baumannii* 6B92 and the elimination of carbapenem resistance, with the subsequent loss of this enzyme, the work suffered from the inability to transfer the resistant gene into a well-characterised host. This would have allowed the contribution of OMP changes or alteration in affinity for PBPs to be examined. It could be argued therefore, that the ARI-1 ß-lactamase only helps contribute to carbapenem resistance in concert with other factors. This problem was circumnavigated by the utilisation of the ß-lactamase inhibitor BRL 42715. Use of this agent inhibited the activity of any ß-lactamase and demonstrated that without the protective action of ARI-1 the strain succumbed to the carbapenems, with MIC levels of the parent strain, falling to almost the same values as those for the cured strain, thus, confirming the importance of ARI-1 in the contribution of carbapenem resistance in *A. baumannii* 6B92.

3.8. The significance of ARI-1

Serine active site carbapenemases are perhaps the rarest of all the ß-lactamases. The only other reports of ß-lactamases that are not inhibited by EDTA (i.e. enzymes which are presumed not to have a zinc centred active site), with carbapenem hydrolysing properties, are those of the NMC-A ß-lactamase described by Nordmann *et al.* from *E. cloacae* NOR-1 [152,254], and very recently, SME-1 (pI 9.7), from *S. marcescens* S6 [153]. Both enzymes have been sequenced [153,254] and are Ambler Class A ß-lactamases. SME-1 was initially inferred to be a metallo-ß-lactamase on the basis of

data suggesting inhibition by EDTA [154], however the bla_{SME-1} gene shows no homology with the Class B metallo-ß-lactamases, but did show 70% identity with NMC-A. The NMC-A ß-lactamase shares several similarities with ARI-1, i.e. the pIs are similar (NMC-A=6.9), and both enzymes preferentially attack penicillins, carbapenems and cephaloridine, although NMC-A did confer some resistance to expanded spectrum cephalosporins and was also partially inhibited by clavulanate.

There has also been an enzyme reported from a strain of *B. distasonis* TAL 7860 [150]. The enzyme from *B. distasonis* has a pI of 6.9 and a M_r of >60kDa which suggests that it differs from ARI-1. However full biochemical characterisation is unavailable for this enzyme and a valid comparison is not possible.

ARI-1 is unlikely to have been selected by antibiotic usage, therefore it is interesting to speculate on the origin of this enzyme. It is vital therefore, that the gene encoding for ARI-1 is cloned into a suitable shuttle vector with subsequent sequencing, only then will we be able to ascertain whether ARI-1 has evolved from a point mutation of a known gene or whether we have discovered a new class of ß-lactamase, previously undetected. It would be interesting to perform hybridisation experiments with a probe constructed from the NMC-A gene, which has been cloned into a plasmid vector and expressed in *E. coli*. Hybridisation experiments with a 1.2 kb fragment from the recombinant plasmid harbouring the structural gene for NMC-A failed to hybridise with any known ß-lactamase. Although, the hybridisation experiments did not include a gene probe for SME-1.

Whatever the origin of ARI-1, our results intimate that the enzyme has almost certainly been introduced into *A. baumannii* 6B92 from an extraneous source. This implies that the ß-lactamase is already in the general population of organisms and has been for some time. If the gene has transposed into the genome of *A. baumannii*, it would seem logical to assume that the same transfer would occur in other, more clinically relevant species. With the increase in use of carbapenems, this would allow these resistant organisms to be selected, as has occurred with the ESBLs, particularly in intensive therapy units (ITUs) in France and the USA. This would greatly compromise our ability to treat nosocomial infections for which imipenem is, often, the last efficient ß-lactam for use in patients in ITUs.

4.0. Investigation of the susceptibility pattern and the ß-lactamases of the species, *X. maltophilia*

4.1. Antimicrobial susceptibilities of *X. maltophilia*

There was a significant disparity between the susceptibility data obtained with the two different sensitivity test media employed. In general, there was a higher degree of resistance demonstrated to those strains examined on MHA rather than those on ISTA, this was particularly apparent with the ß-lactams. The results with ciprofloxacin and gentamicin were more in agreement. This phenomenon has previously been well publicised, and our results are consistent with work reported by other authors [205,226-228].

Our understanding of the susceptibility behaviour of *X. maltophilia* on differing media has been clarified (but not fully explained) by previous studies. There have been several attempted explanations for this occurrence. Akova *et al.* [228], reported that this effect was independent of ß-lactamase production. Their studies with mutations of *X. maltophilia* expressing low and high levels of L1 and L2 ß-lactamase, demonstrated that these medium-determined effects were independent of the mode of expression of ß-lactamase, and that this behaviour may reflect a permeability difference between cells grown on different media. Conversely, Hawkey *et al.* [226] reported that the degree of resistance displayed by *X. maltophilia* strains to imipenem, and other ß-lactams, correlated positively with the zinc content of the test media employed. The authors assumed that this increased resistance was as a result of increased activity by the zinc dependent L1 enzyme.

Bonfiglio and Livermore [255] argued against the latter hypothesis on several counts.

1. *X. maltophilia* isolates were more resistant to ß-lactams when tested on different formulations of media in which the nutrient concentration had been reduced, with a concomitant reduction in zinc concentration [227].

2. Their own observations showed that cefsulodin MICs to a variety of strains were lower on IST than MH agar. This would be independent of L1 activity because cefsulodin has been shown to be stable to L1 [123].

3. Imipenem MICs have been shown to be less affected by the test medium than were those of other ß-lactams [228]. The opposite pattern would be expected if the zinc concentration were critical.

They too postulated that changes in permeability were the most likely explanation, although they did state that the effect of zinc content in the media formulation should not be entirely discounted.

These previous studies have extended our understanding on the susceptibility behaviour of *X. maltophilia* but have not offered a complete explanation. We have not attempted to resolve this puzzling occurrence in this thesis, suffice to say that susceptibility testing of *X. maltophilia* has been shown to be a difficult task, and extreme caution should be exercised in the interpretation of *in vitro* assay results in correlation with the clinical performance of ß-lactams.

The above findings pose the question, "Which medium more correctly reflects the susceptibility situation *in vivo*?" Hawkey *et al.* [226], suggested that the concentration of the test medium be monitored to reflect the normal serum levels of zinc ions (11-20 µmol). Realistically, this form of quality control would be difficult to establish in the routine laboratory, the same workers also suggest that exogenous levels of zinc ions from such sources as metal caps with rubber washers be controlled!

Most recent studies have employed MH agar as the test medium [200,201,256], and our MIC results correlate well with those findings. Generally, on MH agar, all isolates of *X. maltophilia* were resistant to most antibiotics tested, with no particular drug, or class of drug exhibiting any consistently useful activity. *X. maltophilia* is an up and coming pathogen, especially among those patients with a compromised immune system, therefore, the lack of a suitable antimicrobial for these patients is an obvious concern.

Although not examined in this present work, other workers have demonstrated useful *in vitro* activity with combination therapy, employing either trimethoprim/sulphamethoxazole, aztreonam/clavulanate or ticarcillin/clavulanate [200,257]. Vartivarian *et al.*, [257] demonstrated that the new investigational quinolones PD 117558, PD 117596 and PD 127391 show promise against this difficult organism.

4.2. ß-lactamases of *X. maltophilia*

Any significant ß-lactamase activity exhibited by any of the 28 strains examined required the addition of an inducer prior to extraction of the enzyme. This result was anticipated, previous studies have also shown that enzyme production in this species is inducible [123,202-205]. What was surprising was the diversity of different ß-lactamases produced by the species, with most strains encoding for the production of two or more ß-lactamases. This is contrary to the work of Cullmann and Dick [205] who reported only single enzymes present in each of 20 strains examined. On the basis of pI, it was possible to categorise the ß-lactamases into 12 distinct isoelectric patterns. The induciblility of these enzymes and the failure of the conjugation studies would suggest that these enzymes were all chromosomally mediated. They were thus designated as *Xanthomonas maltophilia* chromosomal enzymes (XMCEs), and were classified (on the basis of pI) into twelve enzyme sub-types.

Only one IEF pattern appeared with any regularity, XMCE type-1. This ß-lactamase pattern was observed in 7 (25%) of the strains examined, and was the only IEF pattern common to more than two strains. Although it should be noted that some isolates did harbour identical ß-lactamases, these were mostly found to have been isolated from the same patient, and were probably the same strain. None of the strains appeared to code for the original L1 and L2 enzymes. The ß-lactamase patterns were so diverse that ß-lactamase profiles would perhaps serve as a useful tool for epidemiological typing, along with other techniques, for *X. maltophilia* species!

There appeared to be no obvious correlation between antibiogram and enzyme type (most strains were resistant to most drugs anyway); however, this would be a difficult comparison to assess, as it is known that permeability is an important consideration in the sensitivity of this species to antimicrobials.

XMCE type-1, was the most prevalent of all enzyme types detected, and strain 5B105 was selected as representative of the group for extensive biochemical examination.

4.3. Characterisation of the XMCE type-1 ß-lactamase from strain 5B105.

All enzyme production in this strain was inducible, with imipenem a more potent inducer of the ß-lactamase than cefotaxime. This is in agreement with other studies [205,228]. No enzyme activity was detected in the culture filtrate, indicating that the ß-lactamase was located in the periplasm.

All ß-lactamases were produced with a single inducer, this might infer that the enzymes share a common regulatory region, although it is known that imipenem is an effective inducer for more than one ß-lactamase [258]. An analogous situation is thought to occur with L1 and L2. Nevertheless, the genetic basis for the linkage of the L1 and L2 enzymes is largely speculation. Dufresne *et al.*[259] successfully cloned the L1 gene into a plasmid vector, complete with considerable flanking sequences, but this did not result in simultaneous production of the L2 enzyme, which would make it highly unlikely that the L1 and L2 genes are located within the same operon.

The production of more than one inducible ß-lactamase is unusual, although they have previously been reported in strains of *Aeromonas* spp. [122], *B. cereus* [145], and *Yersinia enterocolitica* [260]. Some *Aeromonas* spp. have been reported to produce up to three different ß-lactamases [261].

Polyacrylamide gel overlays with various inhibitors demonstrated that the ß-lactamase of pI 6.8 was inhibited by EDTA but not BRL 42715, indicating that this enzyme was distinct from the other bands of activity. Inhibitor overlays also demonstrated that the enzymes of lower pI were inhibited by both BRL 42715 and potassium clavulanate. Aztreonam and cloxacillin overlays had no significant effect on any of the enzymes. These results initially indicated that the enzyme of pI 6.8 was a metallo-ß-lactamase, and the enzymes of pI< 6.8 were broad-spectrum penicillinases, these preliminary findings were to prove surprisingly accurate.

Purification of the ß-lactamase bands of pI <6.8 by electrodialysis and subsequent isoelectric focusing demonstrated that each preparation focused as a single band of activity at exactly the same pI it had originally migrated to. The small yield of enzyme made this technique unsuitable for purification of enzyme for further studies. Native PAGE of the concentrated partially purified ß-lactamase preparation revealed the presence of four distinct bands of activity. These results indicated that the ß-lactamase

presence of four distinct bands of activity. These results indicated that the ß-lactamase activity of pI <6.8 was the result of at least three distinct enzymes and not satellite bands. The differences in pI of these ß-lactamase bands of lower pI suggest that they may have undergone amino acid substitution or addition away from the active site of the enzyme and may physically differ in their primary structure.

When partially purified by gel filtration, the ß-lactamase activity could be separated into two fractions, a ß-lactamase with a pI of 6.8, and the other fractions containing the bands of lower pI (pI<6.8).

The bands of pI< 6.8 that were inhibited by BRL 42715 were eluted at the same rate, with an apparent M_r of 48 kDa. When the M_r was estimated by SDS-PAGE with subsequent renaturation of ß-lactamase activity, only one band was observed with a M_r of 24 kDa, suggesting a dimeric enzyme. It would be improbable that an organism would encode for three or more ß-lactamases with identical molecular masses and sub unit conformation unless they were closely related, like the TEM enzymes, and in parallel with the TEM ß-lactamases the difference in pIs arise from a few amino acid substitutions.

Unfortunately, the identical molecular mass of these enzymes and the similarity of their pIs made separation of these enzymes virtually impossible, despite employing cation and anion exchange chromatography with different pH buffers (i.e. above and below the pI of the ß-lactamases). Not only were the distinct bands of ß-lactamase not separated adequately, the resultant enzymatic activity of the elutate was very poor, and of limited value for further studies. The technique was especially detrimental to the enzyme of pI 6.8.

It was clear that further separation of these enzymes was impossible, and the two fractions separated by gel-filtration were used for further studies. The ß-lactamase of pI 6.8 was designated XM-A. The ß-lactamases of pI< 6.8 were named XM-B.

4.4. XM-A

The XM-A enzyme was very similar in physical and biochemical profile to the L1 enzyme. It was unstable in sodium phosphate buffer. This probably results from the formation of zinc phosphate with the subsequent removal of free Zn^{++} from the buffer. The plasmid-mediated metallo-ß-lactamase from *Ps. aeruginosa* is also

unstable in phosphate buffer [126]. The catalytic activity of the XM-A enzyme was dependant on Zn^{++} for activity. Although restoration of activity with other metal ions was not attempted in this study, the results suggest that the enzyme is a metallo-ß-lactamase. Both enzymes share similar pIs (L1, pI 6.9). In parallel with the L1 enzyme the XM-A enzyme also exists as a tetramer in the active state (M_r 96kDa), with a subunit M_r of 26kDa. Inhibitory profiles for both enzymes are very similar. Both are insusceptible to potassium clavulanate but susceptible to mercuric chloride. Iaconis & Sanders [122], reported that the L1 producing strain that they examined was inhibited by p-CMB (20%), whereas Saino *et al.* [123] reported no inhibition by this compound. Under our test conditions, p-CMB inhibited the enzymatic activity of the XM-A enzyme by 69%. The zinc active sites of metallo-ß-lactamases are thought to include a conserved cysteine residue [119], therefore, inhibition by p-CMB would be expected. As with other metallo-ß-lactamases [58], XM-A, although exhibiting predominantly penicillinase activity, also hydrolysed a broad range of ß-lactams including imipenem but not the monobactam, aztreonam.

It has been shown that the XM-A enzyme shares similar bio- and physiological properties with the L1 enzyme, and clearly belongs in group 3 (metallo-ß-lactamases (MET-N)) in the classification scheme of Bush [149], however full sequencing is essential for an absolute determination.

4.5. XM-B

The other enzymes described in this thesis, XM-B, although differing particularly in isoelectric point to that of the L2 enzyme described by Saino [203] (pI 8.4) shared some similarities with this enzyme. Although it should be noted that the results obtained with the combined enzymes should be treated with caution. It has been shown that these enzymes have distinct physically properties (pI), nevertheless each individual enzyme has not been purified to homogeneity, therefore it has not been unequivocally shown that all these enzymes react in the same manner.

The inhibitor profile for XM-B was similar to that reported for the L2 enzyme. All enzymes were inhibited by clavulanate, p-CMB and mercuric chloride. EDTA had no effect on the enzymatic activity of XM-B indicating that they do not require a divalent metal ion for catalytic activity. Inhibition by BRL 42715 suggests they are serine active site ß-lactamases. The M_r of the enzymes was 48 kDa by gel filtration, whereas their subunit form was estimated to be 24 kDa by SDS-PAGE. The results show they

are dimers in the native state, similar to the L2 enzyme. This enzyme has a M_r of 56kDa when measured by gel filtration and a subunit M_r of 27 kDa when measured by SDS-PAGE. Together, the XM-B group of enzymes hydrolysed a broad spectrum of antibiotics including penicillins and cephalosporins, but not imipenem or aztreonam, whereas the L2 enzyme was primarily a cephalosporinase, with poor hydrolytic activity against the penicillins.

The XM-B group of enzymes appear to be well placed in the Bush Class 2b' (extended broad spectrum ß-lactamases inhibited by clavulanic acid (EBS-Y)) [172], however without cloning of the individual genes into a suitable host and subsequent sequencing of the enzymes, it is not possible to determine whether these enzymes have diverse biochemical profiles.

Neither the XM-A nor the XM-B group of enzymes were shown to hydrolyse aztreonam despite all strains examined exhibiting a high level of resistance to this drug. Other workers have shown both L1 [123] and L2 [203] to hydrolyse this compound. This disparity was also noticed by Felici *et al.* [148]. They failed to show any hydrolysis of this substrate with the L1 enzyme used in their study. Another explanation of the high level resistance to aztreonam may be the low permeability of the outer membrane of X. *maltophilia* to the influx of antibiotics [202].

4.6. Concluding remarks

Although the genetic location of the genes which encode these ß-lactamases has not been precisely determined, the inducibility of the enzymes would suggest that they are chromosomally mediated. Indeed from this study and others [33,205], there appears to be a paucity of plasmid mediated ß-lactamases in strains of *X. maltophilia*.
The XM-A and XM-B enzymes may be common amongst strains of *X. maltophilia*. Cullmann & Dick [205] reported the presence of 6 distinct ß-lactamases in 19 strains of *X. maltophilia*, on close scrutiny of the IEF polyacrylamide gel there appears to be ß-lactamases from strain 858 that are similar to those described in this report, although Cullmann does not describe these enzymes in the text. Assuming that Cullmann's strain 858 did code for enzymes similar to XM-A and XM-B, then XM-A has not been visualised. Several of the strains examined in this study demonstrated multiple ß-lactamases only after prolonged staining. Payne *et al.* [33] reported similar results in a recent study, and demonstrated that in some of their strains, metallo-ß-lactamases were visualised only after the gel was overlaid with zinc ions prior to

ß-lactamase, and that prolonged staining or overlaying the gel with zinc ions prior to staining might have resulted in the visualisation of other enzymes. Further evidence for this hypothesis stems from the fact that Cullmann was unable to shown any inhibition by EDTA for any of his ß-lactamases, and suggested a new class of carbapenem hydrolysing ß-lactamase, not inhibited by EDTA, and therefore not metallo-ß-lactamases. He does not mention what reporter substrate was used for the inhibitor assays, but if a substrate was used that was hydrolysed more efficiently by the serine ß-lactamase rather than the metallo-ß-lactamase (assuming that all *X. maltophilia* strains produce serine and metallo-ß-lactamases), e.g. nitrocephin, then he was simply demonstrating hydrolysis of the reporter substrate by the serine active site ß-lactamase, which would not have been inhibited by EDTA, rather than inhibition of the metallo-ß-lactamase.

Recently, Payne *et al.* [33] also reported enzymes with similar pIs in two strains of *X. maltophilia*.

It appears that within the species *X. maltophilia* a number of different ß-lactamases are produced which differ from the ß-lactamases of most other genera.

5.0. PCR-amplification of genomic DNA from X. maltophilia

The similarities of the physical and biochemical characteristics of the XM-B enzymes to the TEM enzymes did not go unnoticed; similar isoelectric points, subunit M_r, substrate profile and inhibition by potassium clavulanate. However, PCR of the genomic DNA of *X. maltophilia* 5B105 with universal TEM primers, revealed no amplification of TEM DNA, and hence no significant homology between the two enzymes. Although an interesting hypothesis, the result was not unexpected. It has been previously thought that the TEM-1 ß-lactamase evolved from LEN-1, the chromosomal ß-lactamase of *K. pneumoniae*. This hypothesis was strengthened by Huletsky *et al.* [67], who made significant inroads toward answering the question of how the Class A ß-lactamases evolved by studying the phylogeny of the SHV-2 ß-lactamase. They recognised two major groups of proteins, the ß-lactamases of Gram positive bacteria, and the ß-lactamases of Gram negative bacteria. In the Gram negative group, the PSE and CARB enzymes branched off early, followed by LEN-1, and OHIO-1. SHV-1 and SHV-2 branch off from OHIO-1, and are separated by a very short evoluntionary distance. The TEM enzymes are situated close to the SHV

family, indicating that they are highly related to this group. This thesis has also been supported by the work of Arakawa *et al.* [262]. Although sequence comparisons show that LEN-1 shares only 67% amino acid homology with TEM-1 and SHV-1, the sequence encompassing the active site is highly conserved, with 22 of 24 residues identical [263].

6.0. Utilisation of selective pressure on E. coli with imipenem.

Selective pressure on *E. coli* with imipenem, failed to select any mutations with the ability to hydrolyse carbapenems. There was a slight increase in imipenem resistance but no concomitant change in ß-lactamase production. This slight increase is probably as a result of change in outer membrane permeability. No TEM-derived ß-lactamase to date has been shown to hydrolyse carbapenems. This resistance to most ß-lactamases stems from the *trans* configurations of the side chains in the carbapenem molecule rather than the *cis* configuration of other ß-lactams [29].

At present, despite the steady world-wide increase in carbapenem usage, these enzymes appear to be unable to affect the efficacy of the carbapenems.

7.0. Epilogue

The primary aim of this thesis was to study the impact of the introduction of the more powerful ß-lactams on the susceptibility of a specific group of organisms isolated over a 12 year period, with particular interest in the sensitivity of these strains to the newer carbapenems. It is a concern that the incidence of resistance to the third generation cephalosporins is probably higher than was anticipated. The extremely low incidence of resistance to the newer carbapenems was reassuring although the discovery of the ß-lactamase ARI-1 from a strain of *A. baumannii* is a concern. It demonstrates that these enzymes do exist in the general bacterial population, and may be selected with more extensive usage of carbapenems. Carbapenems are currently employed as a "last line" defence in the battle against infection, and the spread of those organisms able to resist these drugs would greatly compromise our ability to treat serious bacterial infections in the future.

It was also shown that the ubiquitous bacterium, *X. maltophilia* produces a variety of ß-lactamases that hydrolyse almost the entire spectra of ß-lactams. The increased use of ß-lactam drugs, particularly the newer carbapenem compounds, may facilitate the emergence of this organism as an important pathogen. If the genes encoding these enzymes acquire the capability to disseminate among the more common pathogens, it will further diminish the range of antibiotics that are available for use in cases of serious sepsis.

8.0. Directions for future research

The work in this thesis has perhaps allowed an insight into the types of ß-lactamase that may prove to be of greater clinical concern in the years to follow.

However, there is still work to be performed.

8.1. Screening

- It is vital that adequate screening programmes on the incidence of resistance of Gram negative aerobic bacilli to various antimicrobials and subsequent analysis of the resistance mechanisms to those strains be continued. Only by this mechanism can we monitor future patterns of developing resistance.

8.2. ARI-1

- It is crucial that the gene encoding for ARI-1 be cloned into a suitable plasmid vector and the nucleotide sequence determined. This would allow the molecular class and the evolutionary position of this gene to be determined.

- X-ray crystallography of the protein would provide information on the structure-activity relationship of the enzyme. Such information would increase our knowledge of the resistance mechanism of ARI-1 and help in the development of more effective antimicrobials.

8.3. *X. maltophilia* XMCE type-1 ß-lactamases

8.3.1. XM-A

- The physical and biochemical properties of XM-A are similar to those of Ll, however, only determination of the N-terminal sequence would allow a more precise comparison. The L1 gene has been cloned into *E. coli* but at present there is no information on the nucleotide sequence.

8.3.2. XM-B

- Only purification of the enzymes to homogeneity (which has been shown to be difficult), would allow more precise kinetic and biochemical studies to be performed.

- Subsequent cloning and sequencing of the individual genes to establish the nucleotide sequence(s) would allow examination of the molecular relationship between these enzymes and confirm whether they were diverse enzymes, or related enzymes that differ by only one or two amino acids in their primary structures.

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Cefuroxime-resistant isolates (mg/l)

B/C no	Isolate	CTX	CXM	TOB	NET	GENT	AMP	PIP	CAZ	IMP	MERO	CIPRO	ß-lactamase
1B104	*E. coli*	0.12	8	1	1	0.5	>256	0	0	0.06	0.008	0.03	Chr+TEM-1
1B39	*E. coli*	4	32	1	2	0.5	>256	0	0	0	0	0	Chr+TEM-1
1B4	*E. coli*	4	64	1	1	1	>256	0	0	0	0	0	Chr+TEM-1
1B51	*E. coli*	32	64	1	4	128	>256	0	0	0	0	0	Chr+TEM-1
1B7	*E. coli*	8	64	0.5	2	2	>256	0	0	0	0	0	Chr+TEM-1
1B93	*E.agglom.*	2	64	0.25	0.25	0.12	16	0	0	0	0	0	SHV-1
2B64	*Klebsiella spp.*	0.12	8	0.5	0.25	0.5	>256	0	0	0.25	0.03	0.06	SHV-1
3B113	*E.cloacae*	0.5	16	8	16	128	>256	0	0	1	0.12	0.06	Chr+TEM-1
3B28	*E. coli*	0.5	16	1	1	0.5	>256	0	0	0.5	0.03	0.03	Chr+TEM-1
3B56	*E. coli*	0.25	64	4	1	0.5	>256	0	0	0.25	0.016	0.03	SHV-1
3B91	*E. coli*	<0.12	8	1	1	0.5	>256	0	0	0.06	0.008	0.03	TEM-1
3B93	*E. coli*	<0.12	8	1	1	0.5	16	0	0	0.25	0.016	0.06	Chr
3B95	*E.cloacae*	0.25	16	8	16	128	>256	0	0	0.25	0.03	0.06	Chr+TEM-1
3B96	*E. coli*	0.25	8	8	16	128	>256	0	0	1	0.06	0.06	Chr
4B115	*E. coli*	<0.12	8	2	1	0.5	16	0	0	0	0	0	SHV-1
4B118	*E.cloacae*	0.25	8	2	0.5	0.5	256	0	0	0	0	0	Chr
4B127	*E.cloacae*	8	32	0	0	0.5	64	0	0	0.12	0.016	0.03	Chr
4B169	*E. coli*	>256	>256	0	0	128	>256	0	0	0	0	0	SHV-1
4B186	*Acinetob. spp.*	16	32	0	0	0.5	64	0	0	0.06	0.25	1	Chr
4B53	*K. spp.*	<0.12	8	0	0	0.5	>256	0	0	0.25	0.008	0.06	Chr
4B85	*E. coli*	0.25	8	2	1	0.5	16	0	0	0	0	0	Chr
5B101	*E. coli*	0.25	8	1	1	0.5	>256	0	0	0.03	0.008	0.008	TEM-1
5B116	*A. anitratus*	16	32	0.5	1	0.5	128	0	0	0.03	0.06	0.25	Chr
5B184	*E.cloacae*	0.5	8	0	0	1	>256	0	0	1	0.06	0.06	NONE
5B186	*E.cloacae*	0.5	16	0	0	1	>256	0	0	1	0.03	0.06	Chr

Cefuroxime-resistant isolates (mg/l)

B/C no	Isolate	CTX	CXM	TOB	NET	GENT	AMP	PIP	CAZ	IMP	MERO	CIPRO	ß-lactamase
5B187	*E.cloacae*	0.5	8	0	0	1	>256	0	0	1	0.06	0.06	Chr
5B189	*E.cloacae*	0.5	8	0	0	1	>256	0	0	1	0.06	0.06	Chr
5B203	*E.cloacae*	0.5	16	0	0	0.5	>256	0	0	1	0.016	0.016	Chr
5B206	*E.cloacae*	0.5	32	0	0	0.5	>256	0	0	0.25	0.06	0.016	Chr
5B207	*C. freundii*	2	16	0	0	0.5	>256	0	0	0.25	0.06	0.06	Chr
5B221	*E.cloacae*	8	>256	0	0	0.25	>256	0	0	0.5	0.06	0.03	Chr+TEM-1
5B227	*E. coli*	0.5	16	0	0	1	>256	0	0	0.5	0.03	0.03	Chr
5B239	*S. marcescens*	2	128	0	0	1	>256	0	0	0.06	0.008	0.25	Chr
5B245	*S. marcescens*	2	128	0	0	1	>256	0	0	0.03	0.008	0.25	Chr
5B33	*K. spp.*	0.25	8	8	16	128	>256	0	0	0.25	0.03	0.12	SHV-1
5B43	*A. anitratus*	32	128	32	4	64	>256	0	0	0.06	0.25	0.5	Chr
5B49	*E. coli*	0.5	8	16	16	64	>256	0	0	0.06	0.008	0.03	TEM-1
5B55	*C. freundii*	0.25	8	2	0.5	0.5	>256	0	0	0.12	0.016	0.03	Chr+TEM-1
5B57	*E.cloacae*	0.5	16	16	16	128	>256	0	0	0.12	0.016	0.06	Chr+TEM-1
5B59	*E.cloacae*	128	>256	0.5	0.5	0.25	>256	0	0	0.12	0.016	0.06	Chr
5B90	*E. coli*	<0.12	8	0.5	0.5	0.25	>256	0	0	0.03	0.008	0.016	TEM-1
5B94	*E. coli*	1	16	1	1	0.5	256	0	0	0	0	0	Chr
6B100	*A. lwoffii*	4	16	1	2	1	32	0	0	0.06	0.12	0.25	Chr
6B101	*A. anitratus*	8	16	1	2	1	>256	0	0	0.06	0.12	0.25	Chr
6B103	*S. liquefaciens*	0.12	16	1	0.5	0.5	16	0	0	0.06	0.016	0.06	Chr
6B104	*A. anitratus*	1	16	0.5	1	1	32	0	0	0.06	0.016	0.06	Chr
6B114	*S. liquefaciens*	1	16	0.5	2	0.5	64	0	0	0	0	0	Chr
6B117	*S. liquefaciens*	0.12	16	0.5	0.5	0.5	16	0	0	0.25	0.03	0.008	Chr
6B133	*S. liquefaciens*	0.12	16	0.5	0.5	0.5	16	0	0	0	0	0	Chr
6B15	*Acinetob. spp.*	16	32	2	8	2	128	0	0	0.06	0.008	1	NONE

Cefuroxime-resistant isolates (mg/l)

B/C no	Isolate	CTX	CXM	TOB	NET	GENT	AMP	PIP	CAZ	IMP	MERO	CIPRO	ß-lactamase
6B154	*Acinetob. spp.*	8	32	1	2	1	16	0	0	0.06	0.016	4	Chr
6B157	*Acinetob. spp.*	8	32	1	2	1	128	0	0	0.06	0.25	0.5	Chr
6B170	*S. marcescens*	0.5	32	2	2	1	16	0	0	0.25	0.06	0.5	Chr
6B226	*S. liquefaciens*	0.12	32	1	0.5	0.5	32	0	0	0	0	0	Chr
6B230	*A. anitratus*	8	16	2	8	2	64	0	0	0	0	0	Chr
6B232	*S. liquefaciens*	<0.12	16	0.5	0.5	1	8	0	0	0.25	0.06	0.06	Chr
6B242	*E.cloacae*	32	>256	8	8	64	>256	0	0	2	0.25	0.06	Chr+TEM-1
6B252	*S. liquefaciens*	0.25	16	0.5	0.5	0.5	8	0	0	0.25	0.06	0.06	Chr
6B253	*A. anitratus*	16	32	1	1	1	64	0	0	0.12	0.25	0.25	Chr
6B285	*E.cloacae*	8	128	1	1	0.5	>256	0	0	0.25	0.06	0.06	Chr
6B297	*C. diversus*	2	256	1	1	0.5	256	0	0	0.12	0.016	0.016	Chr
6B298	*S. liquefaciens*	8	>256	1	2	1	128	0	0	0.5	0.03	0.016	Chr
6B324	*A. anitratus*	16	64	1	2	1	64	0	0	0.12	0.25	0.5	Chr
6B333	*S. marcescens*	0.25	64	2	2	1	32	0	0	0.5	0.03	0.12	Chr
6B367	*Pr. mirabilis*	8	32	1	1	1	32	0	0	0.12	0.03	0.03	Chr
6B369	*A. anitratus*	16	32	2	2	1	64	0	0	0.06	0.12	0.5	Chr
6B371	*A. anitratus*	16	32	1	2	1	64	0	0	0.06	0.12	0.5	Chr
6B383	*Acinetob. spp.*	16	32	1	2	1	64	0	0	0.06	0.12	0.5	Chr
6B389	*E.cloacae*	0.5	64	1	1	0.5	256	0	0	0.12	0.03	0.12	Chr
6B390	*E.cloacae*	0.5	64	1	1	0.5	256	0	0	0.12	0.03	0.06	Chr
6B391	*E.cloacae*	0.5	64	1	1	0.5	256	0	0	0.25	0.03	0.06	Chr
6B394	*Pr. vulgaris*	<0.12	128	0.5	0.5	0.5	128	0	0	1	0.016	0.06	Chr
6B4	*A. anitratus*	8	32	2	4	1	64	0	0	0.06	0.12	0.5	Chr
6B410	*A. anitratus*	16	32	4	32	1	64	0	0	0.06	0.12	0.5	Chr
6B413	*S. liquefaciens*	<0.12	32	0.5	0.25	0.25	16	0	0	0.5	0.03	0.008	Chr

Cefuroxime-resistant isolates (mg/l)

B/C no	Isolate	CTX	CXM	TOB	NET	GENT	AMP	PIP	CAZ	IMP	MERO	CIPRO	ß-lactamase
6B415	*A. anitratus*	16	32	<0.12	2	8	128	0	0	0.25	0.25	0.25	Chr
6B59	*S. liquefaciens*	<0.12	16	1	0.5	0.5	8	0	0	0.25	0.016	0.016	Chr
6B82	*K. pneumoniae*	0.12	16	0.5	0.5	0.5	8	0	0	0.06	0.016	0.06	Chr
6B90	*E. coli*	8	32	1	1	1	64	0	0	0.06	0.12	0.5	Chr
6B92	*A. baumannii*	16	32	1	4	0.5	>256	0	0	16	32	0.5	Chr+ARI-1
7B103	*S. liquefaciens*	<0.12	8	0.25	0	0.25	8	0	0	0	0	0	Chr
7B111	*Acinetob. spp.*	4	8	16	32	1	8	0	0	0.25	0.12	0.12	Chr
7B181	*A. anitratus*	4	16	0.5	0.5	0.5	32	0	0	0.008	0.008	0.008	Chr+TEM-1
7B238	*Enterob. spp.*	8	16	1	1	0.5	64	0	0	0	0	0	Chr
7B246	*A. anitratus*	8	32	1	1	0.5	64	0	0	0	0	0	Chr
7B294	*A. anitratus*	8	32	1	1	0.5	64	0	0	0	0	0	Chr
7B339	*E.cloacae*	<0.12	16	<0.12	0.25	0.25	8	0	0	0	0	0	Chr+TEM-1
7B347	*E.cloacae*	8	256	0.5	0.25	0.5	>256	0	0	0.5	0.03	0.06	Chr
7B369	*E. coli*	<0.12	8	0.5	0.5	0.25	>256	0	0	0.12	0.008	0.03	Chr
7B441	*Enterob. spp.*	4	>256	0.5	0.25	0.25	>256	0	0	0.5	0.03	0.06	Chr
7B450	*K. rhinosclero.*	<0.12	8	<0.12	<0.12	0	32	0	0	0.25	0.03	0.03	Chr
7B470	*S. liquefaciens*	<0.12	16	4	2	0.5	16	0	0	0.25	0.03	0.12	Chr
7B75	*A. anitratus*	8	32	1	2	1	64	0	0	0.06	0.12	0.12	Chr
8B145	*E.cloacae*	4	32	1	1	32	256	2	4	1	0.03	0.12	Chr
8B226	*E. coli*	2	128	0.5	2	0.5	32	1	0.5	0.25	0.016	0.06	Chr
8B273	*S. marcescens*	<0.12	16	0.5	1	0.5	256	2	0.5	2	2	0.12	Chr
8B311	*E. coli*	4	256	0.5	1	0.5	128	0.5	1	0.5	0.016	0.06	Chr
8B313	*S. marcescens*	0.5	256	2	2	1	256	4	2	1	0.03	0.06	Chr
8B324	*Enterob. spp.*	0.25	32	8	2	2	32	1	2	1	0.03	0.06	Chr
8B328	*S. marcescens*	2	>256	4	2	1	>256	2	4	1	0.03	0.5	Chr

Cefuroxime-resistant isolates (mg/l)

B/C no	Isolate	CTX	CXM	TOB	NET	GENT	AMP	PIP	CAZ	IMP	MERO	CIPRO	ß-lactamase
8B332	*S. marcescens*	0.25	32	4	2	64	16	1	4	0.25	0.016	0.5	Chr
8B342	*A. anitratus*	32	64	4	8	4	128	0	0	0.25	0.12	0.25	Chr
8B343	*E.cloacae*	0.25	8	4	0.5	0.5	32	2	4	0.5	0.03	0.06	Chr
8B354	*A. anitratus*	4	8	1	1	0	64	0	0	0.25	0.12	0.06	Chr
8B41	*Acinetob. spp.*	8	64	1	1	1	128	64	128	0.5	0.5	1	Chr
8B444	*A. lwoffii*	4	8	8	32	1	16	2	1	0	0.008	0.06	Chr
8B465	*A. anitratus*	16	64	1	1	1	128	32	16	0.25	0.25	1	Chr
8B469	*E.cloacae*	0.25	16	1	0.5	0.25	128	2	8	0.5	0.03	0.25	Chr
8B491	*E.cloacae*	16	256	16	16	128	>256	>256	128	0.5	0.06	0.12	Chr+TEM-1
8B516	*E.cloacae*	2	16	1	0.5	0.5	64	1	4	0.5	0.03	0.12	Chr
8B92	*E.cloacae*	8	256	16	16	128	>256	>256	128	1	0.06	0.12	Chr+TEM-1
9B138	*A. anitratus*	8	64	1	4	1	32	32	4	0.12	0.25	0.12	Chr
9B144	*A. anitratus*	16	64	1	2	1	32	64	4	0.12	0.5	0.25	Chr
9B15	*M. morgannii*	<0.12	16	1	1	1	32	1	0.5	1	0.06	0.12	Chr
9B198	*M. morgannii*	2	32	0.5	0.25	0.5	>256	16	4	2	0.03	0.06	Chr
9B205	*E. coli*	1	16	0.5	0.25	0.25	>256	16	16	0.5	0.03	0.5	Chr
9B208	*Acinetob. spp.*	32	>256	0.5	2	1	>256	16	4	0.12	0.25	0.5	Chr
9B218	*C. freundii*	1	16	0.5	0.25	1	>256	4	2	0.25	0.03	0.06	Chr
9B219	*C. freundii*	1	16	0.5	0.25	1	>256	4	2	0.25	0.03	0.06	Chr
9B22	*A. anitratus*	4	8	2	4	0.5	8	8	16	0.12	0.25	0.25	Chr
9B225	*S. marcescens*	0.25	32	1	0.5	1	128	2	0.25	0.5	0.03	0.12	Chr
9B230	*E.cloacae*	8	>256	4	8	64	>256	>256	8	0.25	0.03	0.06	Chr+TEM-1
9B243	*S. marcescens*	<0.12	32	1	1	1	128	2	0.25	0.5	0.03	0.12	Chr+TEM-1
9B252	*A. lwoffii*	8	32	1	4	1	64	16	4	0.12	0.12	0.25	Chr
9B262	*S. marcescens*	<0.12	32	0.5	0.5	0.5	16	2	0.25	2	0.03	0.06	Chr

Cefuroxime-resistant isolates (mg/l)

B/C no	Isolate	CTX	CXM	TOB	NET	GENT	AMP	PIP	CAZ	IMP	MERO	CIPRO	ß-lactamase
9B269	*S. marcescens*	<0.12	16	2	2	1	16	1	0.25	0.25	0.03	0.25	Chr
9B272	*M. morgannii*	<0.12	8	0.5	0.5	0.5	64	0.5	0.25	2	0.06	0.06	Chr
9B308	*S. marcescens*	<0.12	32	1	1	0.5	64	2	0.25	0.5	0.03	0.06	Chr
9B312	*A. anitratus*	8	16	0.5	2	1	32	16	4	0.12	0.12	0.25	Chr
9B322	*A. anitratus*	64	64	4	64	32	64	4	64	0.12	0.12	4	Chr
9B330	*A. anitratus*	8	16	0.5	1	0.5	64	4	4	0.12	0.25	0.25	Chr
9B337	*S. marcescens*	0.5	64	0.5	1	1	128	0.5	0.25	0.25	0.016	0.12	Chr
9B36	*K. pneumoniae*	16	>256	1	0.5	1	>256	8	8	0.5	0.12	0.06	Chr
9B364	*S. marcescens*	0.5	256	1	1	0.5	64	2	<0.12	0.5	0.03	0.12	Chr
9B49	*Ach.xy*	32	>256	2	2	2	8	0.25	2	1	0	1	Chr
9B51	*A. anitratus*	32	64	2	8	2	128	64	16	0.12	0.25	0.25	Chr
10B106	*A. anitratus*	16	32	0.5	1	1	32	16	8	0.016	0.25	0.25	Chr
10B116	*S. marcescens*	<0.12	16	1	1	0.5	256	1	<0.12	0.06	0.016	0.12	Chr
10B188	*S. liquefaciens*	1	256	4	4	1	256	2	0.25	0.06	0.008	0.06	Chr
10B199	*E.cloacae*	1	16	1	1	1	128	4	0.5	0.06	0.016	0.06	Chr
10B196	*Enterob. spp.*	4	64	1	1	1	256	4	2	0.06	0.03	0.06	Chr
10B199	*E.cloacae*	1	16	1	1	1	64	4	0.5	0.12	0.016	0.06	Chr
10B202	*C. freundii*	2	64	8	8	128	>256	>256	2	0.016	0.03	1	Chr+TEM-1
10B212	*E.cloacae*	0.5	16	2	2	1	128	4	1	0.12	0.03	0.06	Chr
10B216	*E.cloacae*	0.5	8	1	1	1	128	2	0.25	0.06	0.016	0.06	Chr
10B243	*C. freundii*	32	256	0.5	0.5	0.5	256	16	16	0.25	0.03	0.06	Chr
10B247	*Pr. mirabilis*	<0.12	8	0	0.5	0.25	16	1	<0.12	1	0.5	0.12	Chr
10B248	*E.cloacae*	0.25	8	0.5	0.5	0.5	32	2	0.25	0.12	0.016	0.06	Chr
10B262	*Pr. penerri*	<0.12	128	0.5	0	0	256	0.5	<0.12	1	0.12	0.06	Chr
10B277	*A. anitratus*	32	64	4	16	16	16	32	8	0.25	1	2	Chr

Cefuroxime-resistant isolates (mg/l)

B/C no	Isolate	CTX	CXM	TOB	NET	GENT	AMP	PIP	CAZ	IMP	MERO	CIPRO	ß-lactamase
10B280	*E.cloacae*	0.25	8	1	0.25	0.5	32	2	0.25	0.5	0.016	0.06	Chr
10B281	*E.cloacae*	0.5	8	1	0.5	1	32	2	0.5	0.5	0.016	0.06	Chr
10B282	*Pr. vulgaris*	<0.12	64	0.5	0.25	0.25	128	1	0.12	0.5	0.03	0.06	NONE
10B300	*C. freundii*	0.25	8	0.25	0.12	0.25	16	2	0.25	0.016	0.016	0.06	Chr
10B310	*E.cloacae*	0.25	8	0.25	0.12	0.25	16	2	0.25	0.5	0.016	0.06	Chr
10B329	*E. coli*	<0.12	8	2	1	2	>256	256	0.25	0.06	0.016	0.06	Chr+TEM-1
10B335	*A. anitratus*	16	32	1	0.5	1	64	32	8	0.06	0.25	0.5	Chr
10B347	*C. freundii*	0.25	8	8	8	64	>256	>256	0.5	1	0.016	1	Chr
10B348	*Acinetob. spp.*	32	32	0.5	1	0.5	64	32	8	0.06	0.25	1	Chr
10B356	*E.cloacae*	4	128	0.5	0.25	0.5	>256	4	2	0.12	0.03	1	Chr
10B361	*E. coli*	128	>256	0.5	0.25	0.25	>256	64	64	0.25	0.06	0.12	Chr
10B373	*E. coli*	<0.12	8	0.5	0.5	0.5	>256	16	<0.12	0.12	0.016	0.06	Chr+TEM-1
10B376	*Enterob. spp.*	1	>256	4	16	64	>256	>256	2	0.25	0.06	0.12	Chr+TEM-1
10B38	*Enterob. spp.*	<0.12	8	1	0.5	0.5	32	2	0.25	0.12	0.03	0.06	Chr
10B53	*Serr. spp.*	0.25	64	0.5	1	0.5	256	2	0.25	0.03	0.03	0.06	Chr
10B79	*S. marcescens*	0.25	256	1	2	0.5	128	2	0.25	0.06	0.03	0.12	Chr
10B89	*Enterob. spp.*	16	256	0.5	0.5	0.5	256	16	16	0.12	0.03	0.12	Chr
10B91	*S. marcescens*	<0.12	32	1	1	1	256	2	0.25	0.016	0.016	0.12	Chr
11B30	*E. coli*	<0.12	8	0	0	1	2	1	<0.12	0.016	<0.12	0.016	Chr
11B32	*E. coli*	0.12	8	0	0	1	>256	>256	0.25	0.25	0.016	0.03	Chr
11B46	*E. coli*	0.5	64	0	0	0.5	>256	>256	2	0.25	0.016	1	Chr+TEM-1
11B65	*A. anitratus*	16	32	0	0	1	16	>256	4	0.25	0.25	0.12	Chr
11B68	*S. marcescens*	0.5	>256	0	0	1	128	4	0.12	0.25	0.016	0.12	Chr
11B75	*E. coli*	2	64	0	0	0.5	>256	64	2	0.06	0.016	1	Chr+TEM-1
11B84	*Acinetob. spp.*	16	32	0	0	1	16	32	4	0.06	0.25	0.12	Chr

Cefuroxime-resistant isolates (mg/l)

B/C no	Isolate	CTX	CXM	TOB	NET	GENT	AMP	PIP	CAZ	IMP	MERO	CIPRO	ß-lactamase
11B99	*A. anitratus*	32	128	0	0	2	32	64	8	0.06	0.25	0.5	Chr
11B103	*A. anitratus*	16	32	0	0	1	>256	128	4	0.25	0.25	1	Chr
11B122	*E.cloacae*	0.5	8	0	0	0.5	32	4	0.25	0.5	0.016	0.03	Chr
11B124	*E.cloacae*	0.5	8	0	0	0.5	32	4	0.25	0.5	0.016	0.03	Chr
11B135	*E.cloacae*	0.25	64	0	0	1	>256	2	0.25	2	0.06	0.03	Chr
11B137	*Acinetob. spp.*	16	32	0	0	0.5	32	32	4	0.25	0.25	1	Chr
11B166	*A. anitratus*	16	32	0	0	0.5	32	16	4	0.25	0.25	0.5	Chr
11B167	*S. marcescens*	0.25	32	0	0	0.5	16	2	0.25	1	0.03	0.03	Chr
11B170	*K. pneumoniae*	0.12	8	0	0	0.5	128	8	25	1	0.016	1	Chr
11B174	*K. pneumoniae*	0.25	16	0	0	0.5	128	16	1	2	0.03	0.5	Chr
11B180	*Enterob. spp.*	0.25	16	0	0	1	32	4	0.5	2	0.03	0.03	Chr
11B183	*C. freundii*	2	64	0	0	1	>256	4	16	0.5	0.016	0.03	Chr
11B185	*E.cloacae*	0.25	8	0	0	0.25	64	2	0.25	1	0.016	0.03	Chr
11B193	*E.cloacae*	16	>256	0	0	0.5	>256	4	2	2	0.03	0.25	Chr
11B218	*E. coli*	<0.12	8	0	0	0.5	>256	>256	0.25	0.25	0.016	0.03	Chr+TEM-1
11B222	*Enterob. spp.*	128	>256	0	0	0.25	>256	>256	32	2	0.06	0.016	Chr
11B225	*C. freundii*	16	>256	0	0	0.25	>256	64	64	0.5	0.03	0.016	Chr
11B230	*E. coli*	<0.12	8	0	0	1	8	1	<0.12	0.25	0.016	0.03	Chr
11B235	*E. coli*	<0.12	8	0	0	5	16	2	0.25	0.5	0.016	0.03	Chr
11B239	*Acinetob. spp.*	4	8	0	0	0.12	32	16	2	0.25	0.5	0.25	Chr
11B259	*E.cloacae*	0.25	8	0	0	0.25	64	2	0.5	1	0.016	0.03	Chr
11B267	*A. anitratus*	8	16	0	0	0.12	<0.12	8	2	0.06	0.25	0.5	Chr
11B283	*Coliform*	16	32	0	0	0.5	64	32	4	0.25	0.25	0.5	Chr
11B288	*E. coli*	0.12	8	0	0	16	16	2	0.5	0.5	0.016	0.03	Chr
11B289	*Acinetob. spp.*	2	8	0	0	0.5	16	4	1	0.03	0.06	0.5	Chr

Cefuroxime-resistant isolates (mg/l)

B/C no	Isolate	CTX	CXM	TOB	NET	GENT	AMP	PIP	CAZ	IMP	MERO	CIPRO	ß-lactamase
11B290	*A. anitratus*	64	128	0	0	1	>256	64	8	0.25	0.5	0.5	Chr
11B298	*Coliform*	<0.12	32	0	0	0.5	128	0.5	<0.12	2	0.06	0.03	Chr
11B319	*A. anitratus*	4	16	0	0	0.5	128	64	4	0.06	0.25	0.5	Chr
11B351	*E. coli*	<0.12	8	0	0	1	>256	64	25	0.12	<0.12	0.03	Chr+TEM-1
12B4	*Coliform*	0.25	16	0	0	0.25	>256	16	1	0.25	0.016	0.5	None
12B17	*E.cloacae*	0.12	8	0	0	0.5	4	2	0.12	1	0.016	0.03	Chr
12B58	*E. coli*	0.25	8	0	0	1	128	4	0.25	0.12	<0.12	0.016	Chr
12B77	*Kleb. spp.*	<0.12	8	0	0	1	128	8	0.5	0.06	0.016	0.25	Chr
12B112	*E. coli*	<0.12	8	0	0	0.5	16	2	0.25	0.12	<0.12	0.5	Chr
12B120	*Serr. spp.*	8	>256	0	0	0.5	>256	8	4	1	0.06	1	Chr
12B123	*M. morgannii*	<0.12	8	0	0	1	128	0.5	<0.12	8	0.06	0.06	Chr
12B133	*E. coli*	<0.12	8	0	0	2	32	8	0.25	0.06	0.016	0.03	Chr
12B154	*K. pneumoniae*	<0.12	8	0	0	1	128	8	0.25	0.25	0.016	0.25	Chr
12B184	*M. morgannii*	<0.12	32	0	0	1	128	0.5	<0.12	4	0.06	0.06	Chr
12B192	*A. anitratus*	32	64	0	0	2	128	32	8	0.12	0.25	1	Chr
12B208	*E. coli*	<0.12	32	0	0	0.5	8	1	<0.12	0.25	0.016	1	Chr
12B218	*S. liquefaciens*	0.12	16	0	0	0.5	32	1	<0.12	0.5	0.03	0.016	Chr
12B231	*E. coli*	<0.12	8	0	0	1	32	4	0.5	0.12	0.016	0.016	Chr
12B232	*M. morgannii*	<0.12	32	0	0	0.5	128	0.5	0.12	2	0.03	0.016	Chr
12B274	*C. freundii*	0.25	16	0	0	0.5	128	8	0.5	0.25	0.016	0.12	Chr
12B321	*E.cloacae*	4	>256	0	0	0.5	>256	4	2	0.25	0.03	1	Chr
12B327	*Enterob. spp.*	0.25	16	0	0	0.5	64	2	0.5	0.5	0.016	0.03	Chr
12B329	*K. pneumoniae*	128	>256	0	0	1	128	8	2	1	0.06	0.06	Chr+SHV
12B330	*E.cloacae*	4	128	0	0	1	64	4	0.5	0.5	0.016	0.06	Chr
12B336	*A. anitratus*	16	32	0	0	1	128	16	4	0.12	0.25	4	Chr

Cefuroxime-resistant isolates (mg/l)

B/C no	Isolate	CTX	CXM	TOB	NET	GENT	AMP	PIP	CAZ	IMP	MERO	CIPRO	ß-lactamase
12B341	*E.cloacae*	0.25	16	0	0	1	64	4	0.5	0.5	0.016	0.06	Chr
12B362	*A. anitratus*	16	32	0	0	0.5	32	32	4	0.25	0.25	1	Chr
12B373	*E.cloacae*	0.25	16	0	0	1	128	8	1	0.25	<0.12	0.25	Chr
12B384	*E. coli*	16	>256	0	0	0.5	128	4	2	0.25	<0.12	0.016	Chr
12B402	*Ach.xy*	>256	64	0	0	128	>256	>256	>256	1	0.5	0.5	Chr
12B404	*M. morgannii*	1	64	0	0	1	>256	16	4	2	0.03	0.016	Chr
12B428	*M. morgannii*	<0.12	8	0	0	2	16	0.25	<0.12	4	0.016	0.03	Chr
11B289	*Acinetob. spp.*	2	8	0	0	0.5	16	4	1	0.03	0.06	0.5	Chr
12B429	*M. morgannii*	<0.12	8	0	0	0.25	16	0.5	<0.12	4	0.03	<0.12	None
12B453	*E. coli*	0.12	16	0	0	1	>256	64	<0.12	0.25	<0.12	0.06	Chr+TEM-1
12B458	*E. coli*	0.12	16	0	0	1	>256	64	<0.12	0.25	<0.12	0.03	Chr+TEM-1
12B471	*S. marcescens*	0.25	32	0	0	1	64	0.5	0.25	2	0.03	0.12	Chr
12B500	*Sal GpD*	0.25	8	0	0	0.25	8	2	0.25	0.5	<0.12	0.06	None
12B508	*A. anitratus*	8	16	0	0	0.5	8	32	4	0.06	0.5	0.25	Chr
12B511	*E.cloacae*	32	>256	0	0	0.25	>256	32	16	2	0.25	4	Chr
12B516	*Enterob. spp.*	16	>256	0	0	0.25	>256	16	16	1	0.016	0.12	Chr
12B528	*Enterob. spp.*	4	>256	0	0	1	128	4	2	1	0.016	0.06	Chr
12B530	*Acinetob. spp.*	32	32	0	0	1	8	32	4	0.06	0.5	1	Chr
12B534	*A. anitratus*	4	8	0	0	0.25	8	32	2	0.06	0.5	0.25	Chr
12B563	*E. coli*	0.12	8	0	0	0.5	8	2	0.25	0.25	<0.12	0.03	NONE

PUBLICATIONS

Presented at the 6th European Congress on Clinical Microbiology and Infectious Diseases (1993), Seville, Spain, Abstract no 1097.

The incidence of antibiotic resistance in Gram negative aerobic bacilli isolated from blood cultures during 1980-91 from a large Scottish teaching hospital.

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Abstract

This study investigated the susceptibility of 1453 Gram negative aerobic bacilli isolated from Edinburgh Royal Infirmary during 1980-91. The isolates were examined for their susceptibility to ß-lactams, aminoglycosides and the fluroquinolone, ciprofloxacin. All strains were identified by API 20E or if non fermenters API 20NE strips. MICs were performed on Oxoid isosensitest agar (IST) with an inoculum of 10^4 cfu/spot. Percentage resistance was calculated by referral to established breakpoint values for each antimicrobial tested. As expected Escherichia coli was the most prevalent species present (51%), with Klebsiella and Pseudomonas spp the next most prevalent (10%). Organisms which are known to produce a Class 1 chromosomal cephalosporinase constituted approximately 20% of the total number of number of species present. Overall resistance to ampicillin was 63%. Of the three aminoglycosides tested gentamicin was the most effective (12% resistance), whereas resistance to both netilmicin and tobramycin was around 20%. Resistance to the second generation cephalosporin, cefuroxime was high (32%). The third generation cephalosporins ceftazidime and cefotaxime displayed 21% and 16% resistance respectively. The carbapenem imipenem (4%) and ciprofloxacin (3%) were the most efficacious.

Presented at the 33rd ICAAC, New Orleans, USA (1993)

Four Novel Discrete ß-lactamases Responsible for Imipenem Resistance in *Xanthomonas maltophilia*

Abstract

The ß-lactamase complements of 18 strains of *Xanthomonas maltophilia* isolated from blood cultures during 1980-91 from Edinburgh Royal Infirmary were examined by isoelectric focusing (IEF). Seven of the 18 strains (40%) exhibited identical IEF patterns and revealed the presence of at least four main bands of ß-lactamase activity (pI 6.8, 6.2, 5.55, 5.2) when stained with the chromogenic cephalosporin nitrocefin. The ß-lactamase band of pI 6.8 was eliminated by overlaying the gel with filter paper containing 1mM EDTA prior to staining, all other bands were unaffected. It was not clear whether the three bands represented three distinct enzymes or whether they were merely satellite bands of the same enzyme. To elucidate this, each band was cut out of the acrylamide gel and the ß-lactamase was extracted placing the fragment into a dialysis sack and releasing the enzyme in an electric field. Each ß-lactamase was then re purified, applied to an IEF gel and re-separated. Each preparation focused as a single band at exactly the same pI that it had originally migrated to. No satellite bands were found with any of these preparations indicating that the original bands were not satellite bands but discrete ß-lactamases. Between them these ß-lactamases were able to hydrolyse and confer resistance to penicillins, all classes of cephalosporins and imipenem. These results reveal the presence of four previously unidentified ß-lactamases from *X. maltophilia*, one inhibited by EDTA (XMA-1), pI 6.8, and three others which focused at pI 6.2 (XMA-2), pI 5.55 (XMA-3) and pI 5.2 (XMA-4).

Presented at the International Congress on the Management of Infection, Amsterdam, Holland, Abstract No P1.41.

Antibiotic Resistance in Gram Negative Aerobic Bacilli Isolated from Blood Cultures During 1980-1989

R. Paton, J. Hood, R. S. Miles & S. G. B. Amyes

Abstract

This study investigated the susceptibility of 167 aerobic Gram negative bacilli (excluding *Pseudomonas* spp. and *Xanthomonas* spp.) which were isolated from blood cultures in Edinburgh Royal Infirmary from 1980-1989. These isolates had previously been screened for resistance to cefuroxime (MIC> 4mg/l), and this study examined them further for their susceptibilities to other ß-lactams, gentamicin and ciprofloxacin. All isolates were identified by API20E or 20NE. MICs were performed on Oxoid IST (Isosensitest) agar with an inoculum of 10^4 cfu/spot. Percentage resistance was calculated by referral to established breakpoint values for each antimicrobial tested [1]. As expected there was a large number of organisms, such as *Enterobacter* spp. (49) and *Serratia* spp. (32), which produce an inducible cephalosporinase. Despite the fact that *Acinetobacter* spp. do not normally possess an inducible ß-lactamase, there were 43 resistant members of this species in this study. No single antibiotic was completely effective against all species. Imipenem was the most effective with an incidence of <1.0% resistance, resistance to ciprofloxacin was 7.1% while resistance to gentamicin was 13%. All other antibiotics tested showed a high incidence of resistance (>37%). The incidence of gentamicin resistance is disturbing as aminoglycosides are used as frequently as the ß-lactams in the treatment of serious sepsis. Our results show that imipenem and ciprofloxacin remain most effective against these types of organisms.

[1] BSAC Working Party (1991). J. Antimicrob. Chemother. **27** (Suppl. D), 1-50.

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ANTAGE 00033

ARI 1: β-lactamase-mediated imipenem resistance in *Acinetobacter baumannii*

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A strain of Acinetobacter baumannii *6B92 isolated from the blood culture of a patient at the Edinburgh Royal Infirmary in 1985 was found to be resistant to imipenem, all classes of cephalosporins and penicillins. Extraction of the soluble proteins of the cell and isoelectric focusing revealed the presence of two β-lactamases: a chromosomal cephalosporinase of high pI (> pI 9.0) and a novel β-lactamase of pI 6.65 named ARI 1 (*Acinetobacter *resistant to imipenem). Despite the fact that the original clinical isolate could be 'cured' of its resistance to imipenem and penicillins by growing in the presence of ethidium bromide with the concurrent loss of the ARI 1 enzyme, no resistance plasmid was visualised or transferred. The ARI 1 β-lactamase hydrolysed penicillin, ampicillin and cephaloridine slowly during enzyme assay but inactivation of imipenem could only be demonstrated by microbiological means. The molecular size of the ARI 1 enzyme was 23 kDa and it was not inhibited by EDTA, p-CMB, or clavulanate.*

Key words: β-Lactamase; Resistance; Imipenem

Introduction

Acinetobacter spp. are short Gram-negative aerobic bacilli and are important pathogens in nosocomial infections [1]. They have been shown to produce a series of cephalosporinases [2,3] some of which have been particularly difficult to visualise [4]. However, all the cephalosporinases identified to date have been chromosomally-mediated. Plasmid-mediated β-lactamases do exist in *Acinetobacter* spp. but they have always been penicillinases, which have previously been identified in *Pseudomonas* spp. or the Enterobacteriaceae [2]. In addition *Acinetobacter* spp. have developed an impressive pattern of resistance to other antibiotics [5]. In this report we describe a novel β-lactamase enzyme from a strain of *Acinetobacter baumannii* 6B92 isolated in 1985 from a blood culture in Edinburgh Royal Infirmary. This enzyme confers resistance to imipenem, penicillins

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and cephaloridine but not to second and third generation cephalosporins such as cefuroxime, ceftazidime and cefotaxime.

Materials and Methods

Susceptibility testing

All minimum inhibitory concentrations were determined on Isosensitest (IST) agar (Oxoid Ltd., Basingstoke, UK) with an inoculum of 10^4 organisms per spot applied with a Denley multipoint inoculator. Disc sensitivities were performed and interpreted as sensitive, intermediate or resistant by Stokes' method as described by Holt and Brown [6] on IST agar with a rotary plater.

β-lactamase preparation and purification

Cells were grown shaking overnight at 37°C in 9 ml of nutrient broth. This was used to inoculate a 1 l culture of nutrient broth and the cells were grown with shaking at 37°C for 18 h. The cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C. The pellet was washed in 25 mM sodium phosphate buffer (pH 7.0) and recentrifuged at 4000 rpm for 15 min. The pellet was resuspended in 4 ml of the same buffer and disrupted by ultrasonication with constant cooling (8 μm, 1 min × 3, MSE Soniprep 150, MSE Instruments, Crawley, UK). The debris was removed by centrifugation at 12500 rpm at 4° C. The preparation was partially purified by passing it through a G-75 Sephadex gel filtration column (2 $cm^2 \times 90$ cm) (Pharmacia, Uppsala, Sweden). The samples were eluted with 25 mM sodium phosphate buffer. β-lactamase activity was detected by testing each fraction with nitrocefin. Thirty μl of sample was added to 100 μl of 50 mg/l nitrocefin. A colour change from yellow to red indicated the presence of β-lactamase activity.

Molecular mass determinations

Molecular mass (M_r) was determined by the method of Andrews [7]. The molecular weight standards were ovalbumin (45 000), chymotrypsinogen (25 500) and cytochrome-*c* (12 384).

Isoelectric focusing

Samples were applied to a polyacrylamide gel containing ampholines with a pH range 3.5–10.6 (as described by Matthew et al. [8]). The gels were stained by overlaying the surface of the gel with filter paper soaked in nitrocefin (500 mg/l). The β-lactamase activity appeared as red bands on a yellow background.

β-lactamase assays

The enzyme preparation was concentrated before use in a centriprep 10 concentrator (Amicon, Danvers, MA 01923, U.S.A.). Assays of β-lactamase activity were performed at 37°C on a Perkin Elmer Lambda 2 spectrophotometer with freshly prepared antibiotic solutions in 25 mM sodium phosphate buffer (pH 7.0). Values of V_{max} and K_m were derived by linear regression analysis of Lineweaver-Burk plots of initial velocity data at different substrate concentrations.

Inhibition assays

The inhibitory dose for various inhibitors to reduce the enzyme activity by 50% (ID_{50}) was determined by spectrophotometric assay at 37°C. The rate of hydrolysis of nitrocefin (50 mg/l) by the β-lactamase was measured. This procedure was repeated with increasing concentrations of inhibitor until the hydrolysis of nitrocefin was inhibited by 50%.

Genetic studies

Isolation of any plasmid DNA was attempted with three differing techniques [9,10,11]. All these methods are essentially based on alkaline denaturation of chromosomal DNA but with differing procedures for purification and extraction of plasmid DNA. One method has been shown to be especially suitable for isolation of plasmid DNA from *Acinetobacter* spp. [9]. Transfer of resistance was attempted by conjugation to *Escherichia coli* J62-2, *Pseudomonas aeruginosa* PAO18 and a wild strain of *Acinetobacter baumannii* at both 30°C and 37°C. Mobilisation of resistance was attempted with the *inc*P plas-

mid RP4 [12]. Ethidium bromide was used as a curing agent [13]. Transformation of potential plasmid DNA was attempted to *Escherichia coli* C600 [14].

Microbiological assay

This was done by a modification of the technique described by Masuda et al. [15]. The indicator employed was *Staphylococcus aureus* NCTC 6571. Inhibitory zones were determined to the antibiotics to be tested and filter paper discs each containing 15 µl of enzyme preparation undiluted or diluted 1:2, 1:4, and 1:8 in 25 mM sodium phosphate buffer (pH 7.0) were applied to plates seeded with *Staphylococcus aureus* NCTC 6571 so that they would be at the periphery of the expected zone of sensitivity. The plates were incubated at 37°C overnight. Inactivation of antibiotic was indicated by growth of the indicator strain within the expected zone of inhibition.

TABLE 1

Disc susceptibility results and MICs of various antibiotics to both the parent strain and the cured strain

Antibiotic	6B92		6B92 (Cured strain)	
	Disc	MIC⁺	Disc	MIC⁺
Ampicillin	R	>256	I	16
Piperacillin	R	–	I	–
Azlocillin	R	128	I	16
Ampicillin/ Clavulanate*	R	>128	I	8
Cephaloridine	R	–	R	–
Cephalexin	R	–	R	–
Cefuroxime	R	32	R	32
Cefotaxime	I	16	I	8
Ceftazidime	I	4	I	4
Cefoxitin	R	–	R	–
Trimethoprim	R	–	R	–
Gentamicin	S	0.5	S	0.5
Ciprofloxacin	S	0.25	S	0.25
Imipenem	R	16	S	0.12
Aztreonam	R	–	R	–

Abbreviations: R, resistant; I, intermediate; S, sensitive.
*2:1 ratio. The results are expressed in terms of ampicillin.
–, Not tested; +, MIC expressed as mg/l.

Results

Antibiotic susceptibilities

Acinetobacter baumannii 6B92 was resistant to all penicillins tested and resistant or of intermediate sensitivity to all classes of cephalosporins (Table 1) and, surprisingly, resistant to imipenem. The MIC value of imipenem was 16.0 mg/l.

Isoelectric focusing

Isoelectric focusing revealed the presence of two β-lactamases. One enzyme had several satellite bands with a main band focusing at pI 6.65. The other enzyme focused poorly and spread up the gel towards the cathode (Fig. 1).

Molecular mass determinations

Both these enzymes were partially purified and their molecular mass determined by gel filtration. The β-lactamase that had focused sharply at pI 6.65

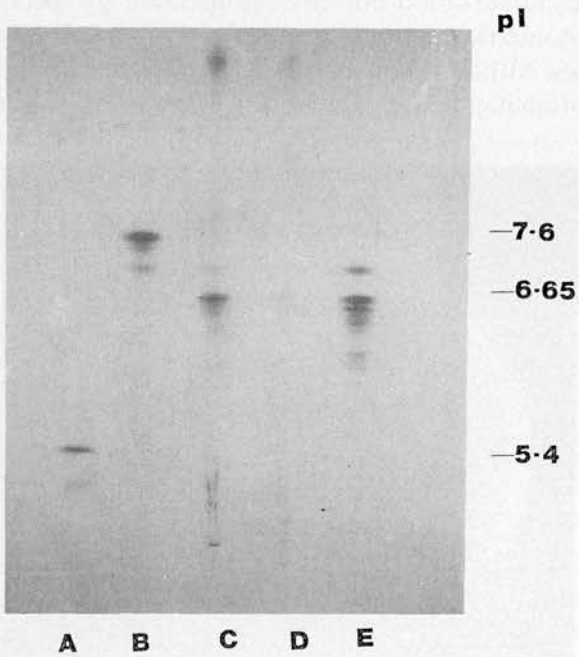

Fig. 1. Isoelectric focusing of *Acinetobacter baumannii* 6B92 β-lactamases. Lane A, TEM 1; B, SHV 1; C, 6B92 parent strain; D, 6B92 cured strain (β-lactamase activity of the enzyme of pI > 9 was stronger in other preparations); E, 6B92 ARI 1 enzyme (purified).

TABLE 2

Hydrolysis of β-lactam antibiotics by ARI 1 enzyme from *Acinetobacter baumannii* 6B92

Substrate	Relative V_{max} [a]	V_{max} [b]	K_m [c]
Penicillin	100	0.128	0.048
Ampicillin	153	0.196	0.139
Cephaloridine	35.7	0.07	0.083

[a]Relative to penicillin 100%.
[b]μmol of substrate hydrolysed/min/ml enzyme solution.
[c]μmol substrate.

had an M_r of 23 kDa whereas the enzyme of pI > 9 had an M_r of 58 kDa.

Hydrolysis of β-lactam antibiotics by the ARI 1 enzyme from *Acinetobacter baumannii* 6B92

The Michaelis constant (K_m) and the maximum rate of hydrolysis (V_{max}) of the pI 6.65 enzyme are shown in Table 2. Penicillin and ampicillin were hydrolysed albeit weakly as was cephaloridine. Hydrolysis of imipenem, cefuroxime, cefotaxime and ceftazidime could not be demonstrated by spectrophotometric methods even after prolonged assay times. Although convenient it is known that spectrophotometry is not suitable for assay of all β-lactamases. Microbiological assay is extremely sensitive and clearly confirmed inactivation of imipenem (Fig. 2) and azlocillin by the pI 6.65 enzyme. Cefuroxime, ceftazidime and cefotaxime were not inactivated. This enzyme was thus designated ARI 1 (*Acinetobacter* resistant to imipenem).

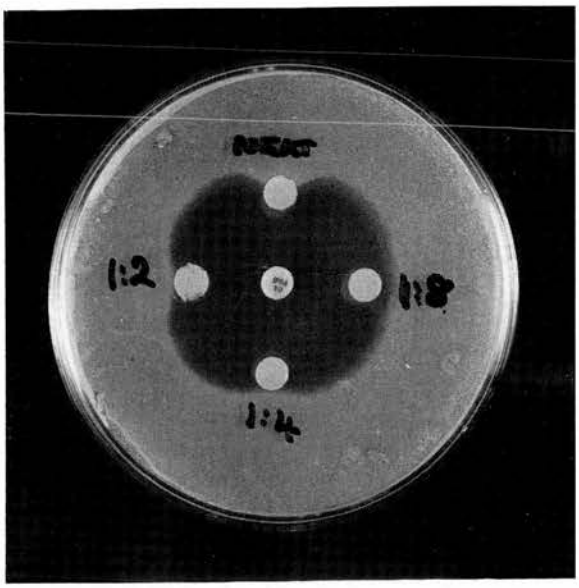

Fig. 2. Microbiological assay plate showing inactivation of imipenem by *Acinetobacter baumannii* 6B92 ARI 1 enzyme.

Inhibitor studies

The effects of inhibitors are shown in Table 3. The ARI 1 enzyme required much higher amounts of clavulanate for inhibition than TEM 1. P-chloromercuribenzoic acid (p-CMB) and EDTA did not inhibit the ARI 1 enzyme. TEM 1 was much less susceptible to inhibition with imipenem than the ARI 1 enzyme. This indicates that the ARI 1 β-lactamase binds imipenem much more strongly than TEM 1.

Genetic studies

Conjugation experiments uniformly could not demonstrate the mobility of the ARI 1 gene and no transfer of plasmid DNA was detected by transformation to *Escherichia coli* C600 (data not shown). However, *Acinetobacter baumannii* 6B92 could be cured of its resistance to imipenem and penicillins. A rate of cure of the resistance gene was obtained by subculturing 100 μl of nutrient broth from the bottle containing the lowest amount of ethidium bromide (in this case 64 mg/l) which visibly inhibited the growth of *Acinetobacter baumannii* 6B92 onto antibiotic free medium. 40 distinct colonies were tested for susceptibility to a range of antimicrobials. The % rate of cure was 10%. No resistance plasmid was visualised or transferred. Once the strain had been cured it contained only the diffuse (presumed chro-

TABLE 3

ID_{50} values of various inhibitors using nitrocefin (50 mg/l) as substrate

Inhibitor	ID_{50} (μM)	
	6B92 (ARI 1)	TEM 1
pCMB	>100	>100
Clavulanate	100	0.5
EDTA	>10000	>10000
Imipenem	0.0875	>100

mosomal) enzyme (Fig. 2). The ARI 1 β-lactamase could not be visualised on IEF which initially suggested that the resistance gene was not located on the bacterial chromosome and was probably situated on a plasmid. When the resistance to imipenem was cured with the subsequent loss of the ARI 1 enzyme the disc sensitivity tests displayed loss of resistance to penicillins and imipenem. This was further confirmed by the MIC data (Table 1) as MICs of penicillins were reduced by a factor of at least 8 and the MIC of imipenem was reduced by a factor of 100. The MICs of second and third generation cephalosporins were not significantly reduced suggesting that resistance to these β-lactams came from the chromosomal β-lactamase of high pI.

Discussion

Carbapenems were initially thought to be resistant to the hydrolytic action of β-lactamases; however, several Gram negative species have been shown to produce enzymes which hydrolyse this group of β-lactams [16–22]. No enzymatic resistance to carbapenems by *Acinetobacter* spp. has been reported so far. Recent studies on resistant strains of *Acinetobacter* spp. selected in the laboratory have indicated that the resistance mechanism involved results from the interplay between penicillin binding proteins with altered expression and/or affinity for β-lactams and decreased permeability of the outer membrane [23,24].

The enzymes which hydrolyse carbapenems are thought to be of chromosomal origin although there have been reports of plasmid mediated metalloenzymes present in *Pseudomonas aeruginosa* [25] and *Bacteroides fragilis* [26]. The production of the novel enzyme described in this report can be cured with the addition of ethidium bromide to the growth medium. Initially this was thought to indicate a plasmid location for the resistance gene; however, no resistance plasmid could be visualised and no transfer of resistance obtained by a variety of methods. It has been shown elsewhere that self transmissible R plasmids transferred from *Escherichia coli* to *Acinetobacter* spp. required a mobilising plasmid for retransfer to occur [27]. Goldstein et al. [28] demonstrated that *Acinetobacter* R plasmids could transfer and express in *Escherichia coli* but at very low frequency. This leads us to the conclusion that the resistance might be chromosomally based and the curing experiments have effected a mutation in the host chromosome. However, this finding does not exclude resistance traits introduced into strains from a plasmid as genetic sequences may be incorporated into the bacterial chromosome, especially from transposons [29] and it is clear that the ARI 1 enzyme is not part of the normal β-lactamase complement of this genus [4].

The enzyme of high pI appears to be a chromosomal cephalosporinase of the type described by Hood and Amyes [30]. These typical *Acinetobacter* enzymes have been shown to focus poorly on conventional isoelectric focusing systems [30].

Hydrolysis of all substrates was very slow and indeed inactivation of imipenem and azlocillin could only be demonstrated by microbiological means. No inactivation of cefuroxime, cefotaxime or ceftazidime was observed. In this instance it is possible that the ARI 1 enzyme functions by a mechanism similar to that in which class 1 cephalosporinases 'trap' the antibiotic [31] by high concentrations of β-lactamase in the periplasmic space; however *Acinetobacter* spp. are not known to produce a class 1 cephalosporinase and Bergogne-Bérézin and Joly-Guillou [5] did not think that typical cephalosporinases produced by *Acinetobacter* spp. were responsible for resistance to imipenem and that the mechanism of resistance in these strains probably results from a selective decrease in permeability across the bacterial outer membrane resulting in decreased rate of antibiotic uptake.

This is the first report of a non zinc dependent carbapenemase from *Acinetobacter* spp. Most carbapenemases contain a zinc ion at their active site which is essential for catalytic activity. Yang et al. [22] reported an enzyme from *Serratia marcescens* that was inactivated by EDTA; however, the addition of zinc sulphate did not restore catalytic activity. Cullman et al. [32] reported non EDTA inhibition in strains of *Xanthomonas maltophilia*. The enzyme described in this report is resistant to inhibition by EDTA, clavulanate and pCMB, but not to imipenem.

There has been a plethora of extended spectrum enzymes [33] reported over the last few years but it

has been shown that these have little effect on carbapenems [34]. Many of these enzymes have been shown to evolve through selective pressure and to be derived from the TEM and SHV family [33]. The ARI 1 β-lactamase has an unusual spectrum of activity, it does not have an extended spectrum profile in that it does not hydrolyse second and third generation cephalosporins, whereas other carbapenemases reported have a wide substrate specificity.

It is interesting to speculate on the origin of this enzyme. The isolate described in this report was isolated in 1985 before carbapenems were in use in our hospital and the concern must therefore be that these enzymes have already been in the general population of organisms for some time. If they are able to disseminate between species they will greatly reduce our capability to treat bacterial infections effectively in the future.

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ANTAGE 00027

Antibiotic resistance in urinary bacteria isolated in central Scotland

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The levels of antibacterial resistance amongst 991 strains responsible for significant bacteriuria, isolated in central Scotland at the end of 1990, have been determined by breakpoint sensitivity testing. Overall resistance to the commonly used antibacterials for UTI, trimethoprim and ampicillin was 23% and 36%, revealing that resistance to these agents in central Scotland had not significantly changed over the last ten years. High levels of ampicillin resistance have led to the widespread use of amoxicillin in combination with the β-lactamase inhibitor clavulanic acid. The effectiveness of this approach was demonstrated by the fact that resistance among these urinary isolates to amoxicillin/clavulanic acid was only 6%. More detailed examination of Escherichia coli *isolates, which were ampicillin-resistant, revealed that the addition of clavulanic acid restored sensitivity in 97.5% of the strains.*

Introduction

Treatment of urinary tract infection represents one of the main uses of antibacterial agents and monitoring the sensitivity of urinary isolates reveals essential information about the changing patterns of antibiotic resistance in a local bacterial population [1–3]. The purpose of this study was to establish the incidence of antibiotic resistance in urinary bacteria isolated in central Scotland. Local factors greatly influence the incidence of resistance and therefore assessments of changes in resistance are more significant if they are made from follow up surveys from the same centres [4]. We compare the results from this survey with similar investigations on specific common antibacterials carried out between 1981 and 1984 [5–7].

Owing to the development of resistance the early mainstays of treatment; namely cotrimoxazole, trimethoprim, nalidixic acid and ampicillin have been joined by many further β-lactam antibiotics, β-lactam/β-lactamase inhibitor combinations and the 4 quinolones. In this study particular attention was paid to resistance to β-lactams and to β-lactam/β-lactamase inhibitor combinations, reflecting the importance of this antibiotic group for treatment of urinary tract infection.

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Materials and Methods

Isolation of causative bacteria

Approximately 1000 consecutive urinary isolates, obtained at the end of 1990 from patients with $>10^5$ organisms/ml, were examined. The specimens were sent to the diagnostic laboratories of the Royal Infirmaries of Glasgow and Edinburgh, cultured onto CLED agar and the causative organism isolated. The organism was identified by the API 20E system unless it was a non-fermentor when it was identified by the API 20NE strip.

Minimum inhibitory concentrations

Minimum inhibitory concentration (MIC) plates were made up with doubling dilutions of the antibacterial drug in Isosensitest Agar (Oxoid, Basingstoke, Hants). Each urine isolate was cultured overnight in nutrient broth and diluted in Davis-Mingioli medium to provide 10^4cfu per µl [8,9]. One µl was inoculated onto the surface of each plate with a Denley multipoint inoculator and the plates were

incubated aerobically at 37°C for 24 h. The amoxicillin plus clavulanic acid determinations were performed at a ratio of 2:1 (amoxicillin:clavulanic acid) and expressed in terms of the amoxicillin concentration [10]. The MIC was taken as the lowest concentration which gave no visible growth.

Antibiotic breakpoint sensitivities

Antibiotic breakpoint sensitivities listed in Table 1 were performed on Oxoid Isosensitest Agar according to the BSAC guidelines [9]. Drug containing plates were checked with a variety of control organisms of known sensitivity.

Results

Overall resistance to the antibiotics tested

The resistance of all the urinary strains was tested at fixed antibiotic concentrations. The total number of bacteria resistant to each antibiotic is shown in Table 1. So that the results may more easily be compared, the percentage resistance has been listed alongside. The highest incidence of resistance was against ampicillin, where nearly 37% of the strains were not susceptible to the drug. However, many diverse species were included in this overall result including *Pseudomonas* spp. and there may be consequent bias. The breakdown of the species involved can be seen in Table 2. Most of the bacteria were *Escherichia coli*, as would be expected in a study of aerobic urinary bacteria such as this.

TABLE 1

The overall resistance of all species tested from both Glasgow and Edinburgh – total 991 strains

Antibiotic	Conc. (mg/l)	Not resistant	resistant (%)
Ampicillin	32	363	36.6
Amoxicillin	32	65	6.6
+ clavulanic acid	16		
Cephalexin	32	146	14.7
Cefuroxime	32	112	11.3
Cefotaxime	16	71	7.2
Ceftazidime	32	70	7.0
Imipenem	4	4	0.4
Nalidixic acid	32	138	13.9
Ciprofloxacin	4	14	1.4
Ofloxacin	16	10	1.0
Gentamicin	8	28	2.8
Nitrofurantoin	32	104	10.5
Trimethoprim	8	230	23.2

Breakdown of bacterial genera or species and the incidence of resistance within each genus

Because of the bias produced by inherently resistant bacteria, the incidence of resistance within each genus of species group was determined. The breakdown of bacterial genera and species and the number of resistant strains is shown in Table 2. The blanks record no resistant bacteria. So that easy comparisons may be made, the percentages are shown in Table 3. As predicted, Pseudomonas had a high incidence of inherent resistance to many antibacterial drugs. Interestingly there were no *Pseudomonas* spp. resistant to ceftazidime or gentamicin and very low resistance to the 4-quinolones was demonstrated. There was a small incidence of resistance to imipenem. The 4-quinolones were less effective against the *Staphylococcus aureus* but all the β-lactam antibiotics were effective. There was no evidence of multi-resistant variants of this species. Streptococci and enterococci remained sensitive to 4-quinolones but, as expected, showed more resistance to cephalosporins . They do, however, remain sensitive to ampicillin.

Ampicillin alone fares less well against members of the Enterobacteriaceae, in particular *Escherichia coli*. The combination of clavulanic acid with amoxicillin rendered most members of this family of bacteria sensitive. This was particularly noticeable with *Escherichia coli* but barely apparent with Citrobacter.

The concentration at which the antibiotic is tested is crucial in the evaluation of the proportion of resistant strains. In Table 4 we have compared the proportion of resistant strains to three cephalosporins at two concentrations. The higher concentration may be more appropriate for the testing of urinary bacteria but would be too high for testing bacteria isolated from other sites. This is an important point as within hospitals there is a well documented spread of resistant organisms and breakpoint testing of urinary isolates will not reveal potential problems such as the presence of extended spectrum β-lactamases.

Comparison of incidences of resistance between Edinburgh and Glasgow

The strains for this study were taken from the two major teaching hospitals in the two major cities of central Scotland. It is known that geographic variation may be important in the incidences of bacterial resistances. Indeed in a previous study carried out in 1984 a considerable difference was found between urinary isolates from Edinburgh and Glasgow with the levels of ampicillin resistance found

TABLE 2

The number of bacteria resistant to each antibiotic within each species tested from both Glasgow and Edinburgh

Species (*N*)	Ampicillin	Augmentin	Cephalex	Cefurox	Cefotax	Ceftaz	Imipenem	Nal. acid	Ciproflox.	Oflox.	Gentamicin	Nitrofuran	Trimethoprim
E. coli (643)	272	10	27	10	–	3	–	25	1	1	5	19	129
Coliforms (3)	2	–	1	1	–	–	–	–	–	–	–	1	–
Proteus (80)	13	4	10	10	–	–	–	1	–	–	–	34	26
Morganella (3)	1	2	3	–	–	–	–	1	–	–	1	–	1
Klebsiella (59)	17	1	3	4	1	–	–	–	–	–	–	7	5
Enterobacter (21)	8	5	8	4	2	–	–	–	–	–	1	3	–
Citrobacter (12)	6	4	5	2	–	–	–	1	–	–	3	–	3
Serratia (2)	1	–	–	–	–	–	–	–	–	–	–	–	–
Pseudomonas (47)	42	39	42	45	30	–	2	34	1	1	–	38	41
Acinetobacter (2)	–	–	2	2	2	–	–	1	1	–	–	2	2
S. aureus (19)	–	–	–	1	1	7	–	13	–	–	–	–	–
Coagulase-negative staphylococci (38)	–	–	–	2	3	12	–	6	8	8	2	–	13
Streptococcus (42)	1	–	25	29	28	31	1	24	1	–	13	–	8
Enterococcus (18)	–	–	18	2	4	17	1	18	2	–	3	–	2
Undefined (2)	–	–	–	–	–	–	–	1	–	–	1	–	–

TABLE 3

The percentage of bacteria resistant to each antibiotic within each species tested from both Glasgow and Edinburgh

Species (*N*)	Ampicillin	Augmentin	Cephalex	Cefurox	Cefotax	Ceftaz	Imipenem	Nal. acid	Ciproflox.	Oflox.	Gentamicin	Nitrofuran	Trimethoprim
E. coli (643)	42.3	1.5	4.2	1.5	–	0.5	–	3.9	0.1	0.1	0.8	2.9	20.1
Coliforms (3)	66.7	–	33.3	33.3	–	–	–	–	–	–	–	33.3	–
Proteus (80)	16.2	5.0	12.5	12.5	–	–	–	1.2	–	–	–	42.5	32.5
Morganella (3)	33.3	66.7	100	–	–	–	–	33.3	–	–	–	–	33.3
Klebsiella (59)	28.8	1.7	5.1	6.8	1.7	–	–	–	–	–	–	11.9	8.5
Enterobacter (21)	38.1	23.8	38.1	19.0	4.8	–	–	–	–	–	4.8	14.3	–
Citrobacter (12)	50.0	33.3	41.7	16.7	–	–	–	8.3	–	–	25.0	–	25.0
Serratia (2)	50.0	–	–	–	–	–	–	–	–	–	–	–	–
Pseudomonas (47)	89.4	83.0	89.4	95.7	63.8	–	4.2	72.3	2.1	2.1	–	80.8	87.2
Acinetobacter (2)	–	–	100.0	100.0	100.0	–	–	50.0	50.0	–	–	100.0	100.0
S. aureus (19)	–	–	–	5.3	5.3	36.8	–	68.4	–	–	–	–	–
Coagulase-negative staphylococci (38)	–	–	–	5.3	7.9	31.6	–	15.7	21.0	21.0	5.3	–	34.2
Streptococcus (42)	2.4	–	59.5	69.0	66.7	73.8	2.4	57.1	2.4	–	30.9	–	19.0
Enterococcus (18)	–	–	100	11.1	22.2	94.4	5.5	100	11.1	–	16.7	–	11.1
Undefined (2)	–	–	–	–	–	–	–	50	–	–	50	–	–

TABLE 4

The differential proportion of resistant bacteria, from both Glasgow and Edinburgh, tested at two different concentrations

Species (*N*)	Cefuroxime		Cefotaxime		Ceftazidime	
	32	8	16	2	32	4
E. coli (643)	10	64	–	5	3	11
Coliforms (3)	1	2	–	–	–	–
Proteus (80)	10	16	–	–	–	–
Morganella (3)	–	2	–	–	–	–
Klebsiella (59)	4	10	1	1	–	1
Enterobacter (21)	4	9	2	4	–	3
Citrobacter (12)	2	5	–	2	–	2
Serratia (2)	–	–	–	–	–	–
Pseudomonas (47)	45	45	30	44	–	11
Acinetobacter (2)	2	2	2	2	–	2
S. aureus (19)	1	1	1	5	7	18
Coagulase-negative staphylococci (38)	2	5	2	12	12	27
Streptococcus (42)	29	29	28	29	31	31
Enterococcus (18)	2	6	4	5	17	18
Undefined (2)	–	–	–	–	–	–

to be 48.5% and 71.9%, respectively [5]. Therefore, we compared the incidences of resistance between Edinburgh and Glasgow, for all the strains (Table 5) and for the *Escherichia coli* strains (Table 6). The results in Table 5 suggest that Glasgow had a slightly more resistant population, except perhaps for nalidixic acid and the fluorinated 4-quinolones. This would be difficult to quantify because of the variation in species distibution. However, when we examined the *Escherichia coli* strains separately (Table 6), the converse appeared to be true, as the bacteria from Edinburgh were invariably more resistant except to ampicillin and trimethoprim.

TABLE 5

Comparison of the incidences of resistance in all strains from Edinburgh (491 strains) and Glasgow (500 strains)

Antibiotic	Number		Percentages	
	Edinburgh	Glasgow	Edinburgh	Glasgow
Ampicillin	162	201	33.0	40.2
Augmentin	27	38	5.5	7.6
Cephalexin	65	81	13.2	16.2
Cefuroxime	32	80	6.5	16.0
Cefotaxime	17	54	3.5	10.8
Ceftazidime	27	43	5.5	8.6
Imipenem	1	3	0.2	0.6
Nalidixic acid	72	66	14.7	13.2
Ciprofloxacin	11	3	2.2	0.6
Ofloxacin	8	2	1.6	0.4
Gentamicin	13	15	2.6	3.0
Nitrofurantoin	47	57	9.5	11.4
Trimethoprim	98	132	20.0	26.4

TABLE 6

Comparison of the incidences of resistance in *Escherichia coli* from Edinburgh (336 strains) and Glasgow (307 strains)

Antibiotic	Number		Percentages	
	Edinburgh	Glasgow	Edinburgh	Glasgow
Ampicillin	125	147	37.2	47.8
Augmentin	6	4	1.8	1.3
Cephalexin	14	13	4.2	4.2
Cefuroxime	2	8	0.6	2.6
Cefotaxime	–	–	–	–
Ceftazidime	3	–	0.9	–
Imipenem	–	–	–	–
Nalidixic acid	20	5	5.9	1.6
Ciprofloxacin	1	–	0.3	–
Ofloxacin	1	–	0.3	–
Gentamicin	5	–	1.5	–
Nitrofurantoin	12	7	3.6	2.3
Trimethoprim	64	65	19.0	21.2

TABLE 7

The different amoxycillin and amoxycillin/clavulanic acid sensitivity phenotypes in *Escherichia coli.*

Minimum inhibitory concentration (mg/l)		Number	Percentage
Amoxicillin	Amoxycillin+clavulanic acid		
1024	64	3	1.2
1024	32	8	3.2
1024	16	86	34.8
1024	8	74	30.0
512	256	1	0.4
512	64	1	0.4
512	32	4	1.6
512	16	2	0.8
512	8	44	17.8
512	4	7	2.8
256	128	1	0.4
256	32	1	0.4
256	16	1	0.4
256	8	1	0.4
256	4	4	1.6
128	32	2	0.8
16	32	1	0.4
8	8	1	0.4
8	4	2	0.8
4	4	2	0.8

Detailed examination of the ampicillin resistant *Escherichia coli* strains

All ampicillin resistant *Escherichia coli* strains were tested for the minimum inhibitory concentration (MIC) of amoxicillin and amoxicillin plus clavulanic acid. The differential results obtained with amoxicillin and the combination revealed a considerable number of different phenotypes. In other words, resistance in the population was not a result of the clonal spread of one bacterial type. In Table 7, the different phenotypes are listed in descending order of their amoxicillin MICs. The results demonstrate the effectiveness of clavulanic acid in restoring amoxicillin resistant strains to sensitivity

Discussion

In this first comprehensive study of general antibiotic resistance in Central Scotland, we have compared the relative sensitivities of a block of clinical urinary isolates to a series of antibiotics. Some of the antibiotics such as ampicillin, amoxicillin plus clavulanic acid, cephalexin, cefuroxime, ciprofloxacin, ofloxacin and trimethoprim are commonly-used against urinary infections. Some of the drugs such as nitrofurantoin and nalidixic acid, despite the low incidences of resistance, are now rapidly falling from favour. The remaining drugs tested ceftazidime and cefotaxime are not currently used against urinary pathogens; however, their inclusion gave a measure of the incidence of resistance in each species to these newer drugs and oral versions of them may soon be used.

The results in this survey show that amongst the *Escherichia coli* the highest incidence of resistances were found to the ampicillin/amoxicillin group and to trimethoprim the resistances being 42.3% and 20.1%, respectively. This is similar to other studies in the UK [1,2,11,12]. However, when amoxicillin was combined with clavulanic acid (tested at a ratio of 2:1 amoxicillin:clavulanic acid), 97.5% of the ampicillin/amoxicillin resistant strains were now sensitive, indicating the continuing efficacy of clavulanic acid to combat β-lactamase mediated resistance to ampicillin. Similar conclusions have been reported by other workers [13]. The sensitivity to the amoxicillin/clavulanic acid combination amongst the *E. coli* was better than trimethoprim and cephalexin and similar to the second-generation cephalosporin cefuroxime. The amoxicillin/clavulanic acid combination was also effective in challenging the ampicillin/amoxicillin resistant strains of *Proteus* spp. and *Klebsiella* spp. It was less effective against *Enterobacter* spp. and virtually without effect on resistant *Pseudomonas* spp., as would be expected. The results showed little difference between the incidences of resistance in Glasgow and Edinburgh, in contrast to earlier studies [5] except that Edinburgh had a slightly greater problem with 4-quinolone resistance and Glasgow with ampicillin/amoxicillin resistance. However, when the Glasgow *E. coli* strains were tested with the combination, there was no significant difference between Glasgow and Edinburgh.

In comparison with our earlier studies it is encouraging to see that there has been no rise in resistance to the common urinary antibacterials trimethoprim and ampicillin. Resistance to trimethoprim in 1981 was 21% in Edinburgh compared with 20% ten years later [7]. Ampicillin resistance in fact appears to have fallen from 48.5% to 33% in Edinburgh and from 79% to 47.8% in Glasgow. Whether this results from a more judicious use of antibacterial agents or simply that there are more antibiotics to choose from and the resistance load is spread more widely is not clear.

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The incidences of resistance to cephalosporins and fluorinated 4-quinolones in similar strains isolated in Glasgow and Edinburgh

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Bacterial sensitivity to cefuroxime, ceftazidime, cefotaxime, ciprofloxacin and ofloxacin was determined for 1386 urinary and bacteraemia isolates from Glasgow and Edinburgh to determine the impact of these antibacterials on the development of resistance. The MIC_{50} and MIC_{90} values were determined for each species or genus. Cefuroxime was the least effective antibacterial drug and cefotaxime was the most potent cephalosporin, but it rarely matched the efficacy of the 4-quinolones. There was little difference in the sensitivities of Gram-negative bacteria from Edinburgh or Glasgow but Gram-positive bacteria isolated in Glasgow were usually more resistant. There has been no significant emergence of resistant Gram-negative bacteria even amongst the Pseudomonas *spp.; however, the proportion of Gram-positive bacteria resistant to these drugs is higher in Scotland than elsewhere.*

Key words: Cephalosporins; 4-Quinolones; Resistance; Bacteremia; Scotland

Introduction

In central Scotland there has been a steady use of cefuroxime, ceftazidime, cefotaxime and ciprofloxacin during the last few years. During the 1980s these drugs represented the ultimate weapons in our arsenal to control multiresistant bacteria [1,2]. Ceftazidime has mainly been used for the treatment of infectious diseases in immunocompromised patients [3] and cefuroxime as an alternative to aminoglycosides to control nosocomial infections [4]. Ciprofloxacin has been the only fluorinated 4-quinolone drug used in this area before the strains were collected. These drugs were genuinely believed to be so sophisticated that no bacterial resistance would emerge in species that are normally sensitive to them. This has, however, been far from the truth. In many areas in Europe and elsewhere, many clinical bacteria have emerged which are resistant to the extended-spectrum cephalosporins [5]. In some part this has resulted from the capability of the plasmid-encoded

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TEM and SHV beta-lactamase genes to undergo simple mutation which allowed the resultant enzyme to hydrolyse all extended spectrum cephalosporins [5]. Similarly clinical strains of staphylococci and pseudomonas have emerged which are resistant to ciprofloxacin [6,7]. Unlike their beta-lactam resistant counterparts, the strains resistant to ciprofloxacin possess mutations exclusively in the chromosomal DNA and the resistance is still not plasmid-mediated [8]. Despite this, however, the problem of resistance is still increasing.

In order to evaluate the impact of the introduction of these drugs on the development of resistance we examined 1386 strains isolated consecutively in the diagnostic laboratories of the two main teaching hospitals in central Scotland, the Royal Infirmaries in Glasgow and Edinburgh.

Materials and Methods

Isolation of causative bacteria

Consecutive significant urine specimens sent to the diagnostic laboratories of the Royal Infirmaries of Glasgow and Edinburgh at the end of 1990 were cultured onto CLED agar and the causative organism isolated. The organism was identified by the API 20E system unless it was a non-fermenter whereby it was identified by the API 20NE strip.

Minimum inhibitory concentrations

Minimum inhibitory concentration (MIC) plates were made up with doubling dilutions of cefuroxime, ceftazidime, cefotaxime, ciprofloxacin and ofloxacin in Isosensitest Agar (Oxoid, Basingstoke, Hants). Each urine isolate was cultured overnight in nutrient broth and diluted in Davis-Mingioli medium to provide 10^4 cfu per µl [9,10]. One µl was inoculated onto the surface of each plate with a Denley Multipoint Inoculator and the plates were incubated aerobically at 37°C for 24 h. The MIC was taken as the lowest concentration which gave no visible growth. Similarly the positive blood cultures were isolated on the Roche slide and these organisms were diluted to provide 10^4 cfu per µl. This dilution was inoculated onto the MIC plates as before. For those bacteria requiring blood, the MIC plates were supplemented with 5% whole horse blood.

Results

At the end of 1990 we examined 975 isolates responsible for significant bacteriuria (491 from Edinburgh and 484 from Glasgow) and 411 strains

TABLE 1

Proportion of specimens and isolates from Edinburgh and Glasgow

	N	
	Edinburgh	Glasgow
Urine isolates	491	484
All Gram-positives	58	50
Streptococcus and Enterococcus	20	32
Staphylococcus spp.	38	18
All Gram-negatives	433	434
Acinetobacter	2	
Citrobacter spp.	10	2
Coliforms	3	
Enterobacter spp.	8	13
Escherichia coli	336	305
Klebsiella spp.	29	30
Proteus and Morganella spp.	28	53
Pseudomonas	15	31
Serratia	2	
Blood isolates	228	183
All Gram-positives	121	114
Diphtheroids	1	5
Enterococcus	12	3
Staphylococcus aureus	53	40
Coagulase-negative staphylococcus	39	49
Streptococcus spp.	16	17
All Gram-negatives	107	69
Acinetobacter	7	5
Citrobacter	2	1
Coliforms	1	
Escherichia coli	52	39
Enterobacter	11	10
Klebsiella	21	12
Proteus	1	
Pseudomonas	10	
Serratia	2	2

TABLE 2

Minimum inhibitory concentrations of extended-spectrum cephalosporins and fluorinated 4-quinolones in Gram-negative urinary bacteria

Antibiotic	MIC (mg/l)					
	Edinburgh			Glasgow		
	Range	50%	90%	Range	50%	90%
All Gram-negatives						
Cefuroxime	<0.008–>128	2	8	<0.008–64	4	32
Ceftazidime	<0.008–128	0.12	0.5	0.015–16	0.25	1
Cefotaxime	<0.008–64	0.03	0.12	<0.008–64	0.03	0.12
Ciprofloxacin	<0.008–32	0.03	0.06	<0.008–1	0.016	0.06
Ofloxacin	<0.008–32	0.12	0.25	<0.008–8	0.12	0.25
Escherichia coli						
Cefuroxime	<0.008–>128	2	4	<0.008–128	4	8
Ceftazidime	0.03–128	0.12	0.25	0.015–16	0.25	0.5
Cefotaxime	<0.008–8	0.03	0.06	<0.008–2	0.03	0.12
Ciprofloxacin	<0.008–16	0.03	0.06	<0.008–1	0.016	0.03
Ofloxacin	<0.008–32	0.06	0.12	<0.008–8	0.06	0.12
Klebsiella spp.						
Cefuroxime	0.25–128	1	4	0.12–128	2	16
Ceftazidime	0.03–0.5	0.06	0.25	0.06–16	0.12	0.5
Cefotaxime	<0.008–0.5	0.015	0.12	<0.008–16	0.03	0.12
Ciprofloxacin	<0.008–0.5	0.03	0.06	<0.008–1	0.03	0.06
Ofloxacin	0.03–2	0.12	0.25	0.03–1	0.12	0.5
Proteus and Morganella spp.						
Cefuroxime	<0.008–>128	0.5	16	0.25–128	1	32
Ceftazidime	<0.008–16	0.03	0.06	0.06–1	0.12	0.25
Cefotaxime	<0.008–0.5	<0.008	0.016	<0.008–0.12	0.015	0.03
Ciprofloxacin	<0.008–2	0.03	0.06	<0.008–0.25	0.03	0.06
Ofloxacin	0.03–2	0.12	0.25	0.03–0.25	0.06	0.25
Pseudomonas aeruginosa						
Cefuroxime	64–>128	>128	>128	1–128	128	128
Ceftazidime	0.5–16	1	16	0.5–8	2	4
Cefotaxime	2–64	8	16	0.06–64	16	16
Ciprofloxacin	0.12–32	0.25	0.5	<0.008–0.25	0.06	0.25
Ofloxacin	0.5–32	1	4	<0.008–4	1	2

isolated from blood culture (228 from Edinburgh and 183 from Glasgow).

Gram-negative isolates responsible for significant bacteriuria

Amongst the urinary isolates, 867 were Gram-negative bacteria of which 641 were identified as *Escherichia coli* (Table 1). Examination of all the Gram-negative bacteria revealed that cefuroxime was considerably less effective than the four other drugs tested; the MIC_{90} for cefuroxime was between 16- and 32-fold higher than it was for the least effective of the other drugs (Table 2). This differential was found within individual species; amongst the *E. coli* strains there was a 16-fold increase in the MIC_{90} of

TABLE 3

Minimum inhibitory concentrations of extended-spectrum cephalosporins and fluorinated 4-quinolones in Gram-positive urinary bacteria

Antibiotic	MIC (mg/l)					
	Edinburgh			Glasgow		
	Range	50%	90%	Range	50%	90%
All Gram-positives						
Cefuroxime	<0.008–>128	1	8	<0.008–128	128	128
Ceftazidime	0.12–>128	16	>128	0.12–128	128	128
Cefotaxime	0.015–>128	1	8	0.015–128	128	128
Ciprofloxacin	0.03–64	0.5	4	<0.008–128	1	2
Ofloxacin	0.12–128	1	4	<0.008–64	1	2
Streptococcus and Enterococcus spp.						
Cefuroxime	<0.008–>128	4	8	<0.008–128	128	128
Ceftazidime	0.12–>128	128	>128	0.25–128	128	128
Cefotaxime	0.015–>128	1	64	0.015–128	128	128
Ciprofloxacin	0.5–4	1	2	0.03–4	1	2
Ofloxacin	1–4	2	4	0.12–4	2	2
Staphylococcus spp.						
Cefuroxime	0.03–>128	0.5	8	<0.008–64	0.5	4
Ceftazidime	0.12–>128	8	32	0.12–128	32	64
Cefotaxime	0.015–128	1	4	0.06–32	1	8
Ciprofloxacin	0.03–64	0.25	64	<0.008–128	0.5	1
Ofloxacin	0.12–128	1	4	<0.008–64	0.25	0.25

cefuroxime compared with the MIC_{90} of ceftazidime.

Ceftazidime was always less active than cefotaxime or the two fluorinated 4-quinolones. There was little to choose between the efficiencies of these three drugs especially in the strains isolated in Edinburgh. Amongst the Glasgow isolates, the MICs of ciprofloxacin were consistently lower than those of cefotaxime and ofloxacin. On the other hand, cefotaxime was at least as effective as ciprofloxacin and rather more effective than ofloxacin when Proteus/Morganella and Klebsiella species were examined individually. The results with the *Pseudomonas* spp showed that only ciprofloxacin remained efficient; almost all the strains were cefuroxime resistant and showed limited susceptibility to cefotaxime (Table 2). There was considerable variation between the ratio of the MICs of ciprofloxacin and ofloxacin, suggesting that the latter drug was more vulnerable to the inherent resistance properties of this species.

Gram-positive isolates responsible for significant bacteriuria

There were 108 Gram-positive urinary isolates (Table 3). Examination of all the Gram-positive bacteria revealed that ceftazidime was the weakest of the five drugs tested. This also showed up when the bacteria were examined in species groups. Amongst the Gram-positive bacteria, the strains isolated in Glasgow were consistently more resistant to the cephalosporins than those isolated in Edinburgh. The results of the species groups suggest that this may result from the Streptococci and Enterococci in the Glasgow population; these isolates seem to have a particularly high incidence of cephalosporin resistance. The fluorinated 4-quinolones were usually more active than the cephalosporins; it was difficult to distinguish between ciprofloxacin and ofloxacin against the Streptococci and Enterococci but ofloxacin was more efficient against the Staphylococci.

Gram-negative bacteraemia isolates

Examination of the Gram-negative bacteria responsible for bacteraemia (Table 4), revealed fewer differences between the efficacies of the drugs than there had been with the Gram-negative urinary bacteria except that cefuroxime was always considerably less effective than the others. At the bottom of the MIC range the Glasgow isolates tended to be more resistant to cefuroxime than those from Edinburgh; the lowest Glasgow MIC was 1 mg/l compared with less than 0.01 mg/l in Edinburgh. This differential stemmed mainly from isolates other than *E. coli*. The sensitivity of the *E. coli* strains to the other four drugs was very similar to the *E. coli* in the urinary isolates. No significant distinction could be made between the drugs; however, amongst the strains overall the two 4-quinolones were more effective than ceftazidime or cefotaxime, with the strains marginally more resistant to ofloxacin than ciprofloxacin.

Gram-positive bacteraemia isolates

The fluorinated 4-quinolones were usually more effective against the Gram-positive bacteraemia isolates than the cephalosporins (Table 5). This was apparent with the Streptococcus and Enterococcus group from Edinburgh as it had with the urinary bacteria; however, the strains from Glasgow appeared more sensitive. The proportion of inherently-resistant enterococci in the Glasgow population was rather lower than in the group from Edinburgh and this probably accounts for the differential. *Staphylococcus aureus* remained susceptible to all the drugs except ceftazidime. This was not the case with the coagulase-negative staphylococci; in both Edinburgh and Glasgow, a considerable proportion of these isolates were resistant to each drug. In Glasgow the coagulase-negative staphylococci were more resistant to the cephalosporins than the Edinburgh isolates had been.

TABLE 4

Minimum inhibitory concentrations of extended-spectrum cephalosporins and fluorinated 4-quinolones in Gram-negative bacteraemia isolates

Antibiotic	MIC (mg/l)					
	Edinburgh			Glasgow		
	Range	50%	90%	Range	50%	90%
All Gram-negatives						
Cefuroxime	<0.008–>128	4	128	1–>128	4	32
Ceftazidime	<0.008–128	0.06	2	0.06–64	0.25	8
Cefotaxime	<0.008–32	0.03	8	0.015–32	0.06	4
Ciprofloxacin	<0.008–2	0.03	0.5	<0.008–0.5	0.015	0.12
Ofloxacin	<0.008–4	0.12	1	0.03–1	0.06	0.12
Escherichia coli						
Cefuroxime	0.5–64	2	4	1–>128	4	4
Ceftazidime	<0.008–2	0.06	0.12	0.06–64	0.25	0.5
Cefotaxime	<0.008–1	0.03	0.06	0.015–16	0.06	0.12
Ciprofloxacin	<0.008–2	0.03	0.06	<0.008–0.03	0.015	0.03
Ofloxacin	0.03–4	0.06	0.12	0.03–0.12	0.06	0.12
Klebsiella spp.						
Cefuroxime	<0.008–16	1	4	1–4	2	4
Ceftazidime	0.03–1	0.06	0.25	0.06–0.5	0.25	0.25
Cefotaxime	<0.008–0.12	0.03	0.06	0.015–0.25	0.06	0.06
Ciprofloxacin	0.03–0.5	0.06	0.12	0.015–0.12	0.06	0.06
Ofloxacin	0.03–2	0.12	0.25	0.06–0.5	0.12	0.12

TABLE 5

Minimum inhibitory concentrations of extended-spectrum cephalosporins and fluorinated 4-quinolones in Gram-positive bacteraemia isolates

Antibiotic	MIC (mg/l)					
	Edinburgh			Glasgow		
	Range	50%	90%	Range	50%	90%
All Gram-positives						
Cefuroxime	<0.008–>128	1	128	0.015–>128	1	32
Ceftazidime	0.03–>128	8	>128	0.03–>128	8	128
Cefotaxime	<0.008–>128	1	16	0.015–>128	2	64
Ciprofloxacin	0.03–64	0.5	4	0.12–>128	1	8
Ofloxacin	0.12–32	0.5	4	0.12–128	1	4
Streptococcus and Enterococcus spp.						
Cefuroxime	<0.008–>128	1	>128	0.016–>128	0.03	2
Ceftazidime	0.03–>128	16	>128	0.03–>128	0.5	4
Cefotaxime	<0.008–>128	1	>128	0.015–>128	0.03	2
Ciprofloxacin	1–8	2	8	2–32	4	16
Ofloxacin	1–8	4	4	2–16	4	8
Staphylococcus aureus						
Cefuroxime	0.5–>128	1	1	0.5–8	1	2
Ceftazidime	8–16	8	16	4–32	8	16
Cefotaxime	0.5–2	1	2	1–16	2	2
Ciprofloxacin	0.12–2	0.5	1	0.5–4	1	2
Ofloxacin	0.12–1	0.5	0.5	0.5–4	1	1
Coagulase-negative staphylococci						
Cefuroxime	0.12–>128	2	32	0.25–>128	1	>128
Ceftazidime	0.25–>128	16	32	4–>128	32	>128
Cefotaxime	0.06–>128	4	16	0.5–>128	4	>128
Ciprofloxacin	0.03–64	0.25	16	0.12–>128	1	32
Ofloxacin	0.12–32	0.5	8	0.12–128	1	16

Discussion

This study has demonstrated that isolates in Central Scotland largely remain sensitive to the extended-spectrum cephalosporins and the fluorinated 4-quinolones. Cefuroxime is less effective against any of the urinary isolates and the Gram-negative bacteraemia strains; however it remains clinically effective in Edinburgh for urinary tract infections. Ceftazidime has lost little of its potency from seven years ago when Gram-negative bacteria from Japan showed very similar resistance patterns to ceftazidime [1]. The MIC_{90} of ceftazidime for *E. coli* in the Japanese study was 0.17 mg/l compared with 0.12–0.5 mg/l found in the urinary and bacteraemia isolates in this study. A more recent survey on strains collected in the USA showed that the MIC_{90} of *E. coli* was 0.25 mg/l [11]; these results suggest that ceftazidime resistance in Scotland, as elsewhere, may not be increasing markedly. Indeed none of the MIC_{90} of ceftazidime for *Klebsiella* spp. in the current study were as high as the 1 mg/l found for the Japanese strains isolated in 1985 [1] or for American strains collected in 1981 [12] or in 1989–90 [11]. On the other hand, the MIC_{90} values of ceftazidime for

staphylococci in the Scottish strains were higher than those obtained in Japan [1].

The trends seen with ceftazidime susceptibility were mirrored with cefotaxime. As would be expected, except in a *Pseudomonas* spp., the Scottish isolates were invariably more sensitive to cefotaxime than ceftazidime and strains from Glasgow were marginally more resistant than those from Edinburgh. The MIC_{90} value of cefotaxime (0.08 mg/l) for *E. coli* found for the Japanese isolates seven years ago matched the range of 0.06–0.12 mg/l found in this Scottish study. On the other hand the Klebsiella strains examined in Scotland were generally more resistant to cefotaxime than their Japanese counterparts. Cefotaxime was the only cephalosporin which approached the efficacy of the 4-quinolones.

Generally ciprofloxacin was more effective than ofloxacin though this trend is often reversed with Gram-positive cocci. This has not been a general phenomenon. In London, the MIC_{90} values of ofloxacin have either equalled or been greater than those of ciprofloxacin and this situation has remained static over five years [2,13]. Elsewhere the MIC_{90} values of ciprofloxacin and ofloxacin for methicillin-sensitive staphylococci have largely remained equivalent [11,14–20].

The MIC_{90} values of ciprofloxacin for *E. coli* ranged between 0.03 and 0.06 mg/l in the current Scottish study. This is rather lower than the level found in London over the past five years [2,13] but was more in line with the susceptibilities found in Switzerland [21] and the USA [11,15,22]. The problem bacteria for the 4-quinolones are *P. aeruginosa* and *S. aureus*. In London, two sequential surveys showed that over a five year period the MIC_{90} of ciprofloxacin for *P. aeruginosa* rose from 0.5 to 4 mg/l although the MIC_{50} did remain constant [2,13]. Our results from Glasgow and Edinburgh show that this species has retained its sensitivity to ciprofloxacin; indeed, they suggest that the susceptibility to ciprofloxacin of *P. aeruginosa* in Scotland was more in line with that found in the USA [11,15,22]. The *S. aureus* strains from the Royal Infirmary Edinburgh are rarely methicillin-resistant; however, more of them appear resistant to ciprofloxacin than the equivalent strains in London [2,13] and elsewhere [16–20,23]. The study in the USA by Fuchs et al. [22] shows a greater proportion of resistant strains but they did not sub-classify the *Staphylococcus* spp. The urinary staphylococci in the present study were not subdivided and a notable number of them were highly resistant to ciprofloxacin in Edinburgh. Extrapolation from the bacteraemia isolates suggests that this may derive from the coagulase-negative staphylococci. The proportion of the coagulase-negative staphylococci resistant to ciprofloxacin in the bacteraemia strains was higher than reported in most centres [2,11,13,16–20,23].

The results from strains isolated in Edinburgh and Glasgow suggest that the Gram-negative bacteria retain their sensitivity to the five drugs tested but the proportion of resistant Gram-positive bacteria may be on the increase.

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Susceptibility of clinical isolates, identified in Edinburgh, to extended-spectrum cephalosporins and fluorinated 4-quinolones

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Introduction

In the Royal Infirmary Edinburgh, there has been a steady use of cefuroxime, cefotaxime, ceftazidime and ciprofloxacin. Ceftazidime has mainly been used for the treatment of infectious diseases in immunocompromised patients. Although ciprofloxacin has been used in the last couple of years, almost no infections have been treated with ofloxacin. In this study, we examined the incidence of resistance to these five antibacterial drugs in bacteria isolated from urinary tract infections and positive blood culture bottles.

Materials and Methods

Each significant urine tested was cultured onto CLED agar and the causative organism isolated. The organism was identified by the API 20E system unless it was a non-fermentor, whereby it was identified by the API 2ONE. The strain was cultured overnight in nutrient broth and diluted to provide 10^4 cfu per 1 µl. Minimum inhibitory concentration (MIC) plates were made up with doubling dilutions in Oxoid Isosensitest Agar. All plates were inoculated with a Denley Multipoint inoculator and were incubated for 24 hours at 37°C. The MIC was taken as the lowest concentration which gave visible growth.

Each positive blood culture was isolated on the Roche slide. This organism was diluted to provide 10^4 cfu per 1 µl and inoculated onto MIC plates. For those organisms requiring blood, the addition of 5% whole blood was included.

Results

In total, 703 clinical isolates were obtained from in-patients in the Royal Infirmary Edinburgh during 1990. Of these 490 came from significant bacteriuria and 213 from bacteraemia. Amongst the urinary isolates 433 were Gram-negative, of which 336 were identified as *E. coli*. (Table 1) The MIC_{90} for cefuroxime was 16 times higher than for ceftazidime and, surprisingly, 64 times higher than cefotaxime (Table 2). The organisms seemed slightly less susceptible to ofloxacin than ciprofloxacin. These differences were not mirrored in the Gram-negative bacteria responsible for bacteraemia (107) where there was a much higher incidence of resistance to cefuroxime (Table 3). Amongst these isolates resistance to cefotaxime was greater than resistance to ceftazidime, although this was not true amongst the *E. coli* (Table 4). Again, there was little difference between ciprofloxacin and ofloxacin but both antibacterial drugs were less effective against these Gram-negative bacteria than they had been against the urine isolates. All drugs were less effective against Gram-positive bacteria, especially ceftazidime, and this mainly stemmed from the coagulase-negative staphylococci (Tables 5 and 6).

Discussion

The results show that Gram-negative bacteria from blood cultures largely remain sensitive to ceftazidime, cefotaxime, ciprofloxacin and ofloxacin. Cefuroxime appeared less effective. In Gram-positive bacteria, ceftazidime has lost some of its potency though most organisms remain susceptible to cefotaxime, ciprofloxacin and ofloxacin.

Table 1. Distribution of species amongst Gram-negative urinary isolates

Acinetobacter	2
Citrobacter	10
Coliforms	3
Escherichia coli	336
Enterobacter	8
Klebsiella	29
Proteus and Morganella	28
Pseudomonas	15
Serratia	2
Total	433

Table 3. Distribution of species amongst Gram-negative bacteraemic isolates

Acinetobacter	7
Citrobacter	2
Coliform	1
Escherichia coli	52
Enterobacter	11
Klebsiella	21
Proteus	1
Pseudomonas	10
Serratia	2
Total	107

Table 2. MICs and ranges for Gram-negative urinary isolates

	Cefotaxime	Cefuroxime	Ceftazidime	Ofloxacin	Ciprofloxacin
All Gram-negatives (433)					
Range	0.015–64	0.06– >128	0.015–128	0.015–32	0.015–32
MIC_{50}	0.03	2.0	0.12	0.12	0.03
MIC_{90}	0.12	8.0	0.5	0.25	0.06
Escherichia coli (336)					
Range	0.015–64	0.06– >64	0.03–128	0.015–32	0.015–16
MIC_{50}	0.03	2.0	0.12	0.06	0.03
MIC_{90}	0.03	4.0	0.25	0.12	0.06
Klebsiella spp. (29)					
Range	0.015–0.5	0.25–128	0.03–0.5	0.03–2.0	0.015–0.5
MIC_{50}	0.015	0.5	0.12	0.12	0.03
MIC_{90}	0.25	128	0.5	0.25	0.12

Table 4. MICs and ranges for Gram-negative bacteraemic isolates

	Cefotaxime	Cefuroxime	Ceftazidime	Ofloxacin	Ciprofloxacin
Gram-negative blood isolates (107)					
Range	0.015–32	0.12– >128	0.015–128	0.03–0.4	0.015–2.0
MIC_{50}	0.03	4.0	0.06	0.12	0.03
MIC_{90}	8.0	128	2.0	1.0	0.5
Escherichia coli (52)					
Range	0.015–1.00	0.5–64	0.015–2.0	0.03–4.0	0.015–2.0
MIC_{50}	0.03	2.0	0.06	0.06	0.03
MIC_{90}	0.06	4.0	0.12	0.12	0.06
Klebsiella spp (21)					
Range	0.015–0.12	0.5–16	0.03–1.0	0.03–2.00	0.03–0.5
MIC_{50}	0.03	1.0	0.06	0.12	0.06
MIC_{90}	0.06	4.0	0.25	0.25	0.12

Table 5. Distribution of species amongst Gram-positive blood isolates

Diphtheroids	1
Enterococci	12
Staphylococcus aureus	53
Coagulase-negative staphylococci	39
Streptococci	16
Total	121

Table 6. MICs and ranges for Gram-positive bacteraemic isolates

	Cefotaxime	Cefuroxime	Ceftazidime	Ofloxacin	Ciprofloxacin
Gram-positive blood isolates (121)					
Range	0.03– >128	0.03– >128	0.03–>128	0.12–32	0.03–64
MIC_{50}	1.0	0.5	8.0	0.5	0.5
MIC_{90}	16.0	32	128	4.0	4.0
Staphylococcus aureus (53)					
Range	0.05–2.0	0.5– >128	8.0– >16.0	0.12–1.0	0.12–2.0
MIC_{50}	1.0	1.0	8.0	0.5	0.5
MIC_{90}	2.0	1.0	16.0	0.5	1.0
Coagulase-negative staphylococci (39)					
Range	0.06–128	0.12– >128	0.25– >128	0.12–32	0.03–64
MIC_{50}	4.0	2.0	16.0	0.5	0.25
MIC_{90}	16.0	32	32.0	8.0	16.0

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Biochemical Properties of Inducible β-Lactamases Produced from *Xanthomonas maltophilia*

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Four different β-lactamases have been found in several strains of *Xanthomonas maltophilia* isolated from blood cultures during 1984 to 1991 at the Edinburgh Royal Infirmary. One was a metallo-β-lactamase with predominantly penicillinase activity and an isoelectric point of 6.8. Its molecular size as determined by gel filtration was 96 kDa but was only 26 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), suggesting a tetramer of four equal subunits. The enzyme hydrolyzed all classes of β-lactams except the monobactam aztreonam. This enzyme was not inhibited by potassium clavulanate or BRL 42715 but was inhibited by *p*-chloromercuribenzoate, mercuric chloride, and EDTA. The β-lactamase was unstable in 50 mM sodium phosphate buffer (pH 8.0) but stable in 50 mM Tris HCl (pH 8.0). The other β-lactamases focused as a series of different isoelectric points, ranging from pI 5.2 to 6.6. Together, these enzymes exhibited a broad spectrum of activity, hydrolyzing most classes of β-lactams but not imipenem or aztreonam. Their molecular size was 48 kDa by Sephadex gel filtration and 24 kDa by SDS-PAGE, indicating that they were enzymes consisting of two equal subunits. They were inhibited by *p*-chloromercuribenzoate, mercuric chloride, potassium clavulanate, and BRL 42715 but not EDTA. This study demonstrated that *X. maltophilia* produces more than just the L1 and L2 β-lactamases.

Xanthomonas maltophilia is the only member of the genus *Xanthomonas* that is pathogenic to humans. This species rests uneasily within this genus, however, and a recent report has proposed that it be placed in a new genus, which includes a single species, *Stenotrophomonas maltophilia* (24). They are glucose-nonfermenting, gram-negative bacilli and are increasingly recognized as an important opportunistic pathogen, often affecting patients with lowered defense mechanisms (12, 21, 22, 29). The species is commonly resistant to a wide range of β-lactams as well as other classes of antimicrobial agents (11, 15, 22). This resistance has been attributed to the interplay between outer membrane impermeability (20) and the production of two potent β-lactamases. L1, a metallo-β-lactamase (31), and L2, described as an unusual cephalosporinase (30), hydrolyze virtually the entire spectrum of β-lactams. It has been assumed that all strains produced the L1 and L2 enzymes. However, recent studies have shown an unexpected heterogenicity among β-lactamases produced by this species (9, 19). Both studies reported several β-lactamases differentiated by their isoelectric points. In the study by Cullmann and Dick (9), none of the strains examined produced more than one β-lactamase. In this study, we report the biochemical properties of β-lactamases produced by seven strains of *X. maltophilia* isolated from blood cultures at the Edinburgh Royal Infirmary during 1984 to 1991.

(Part of this work was presented at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy [25].)

MATERIALS AND METHODS

Bacterial strains. Seven strains of *X. maltophilia* were examined. All strains were isolated from blood cultures taken at the Edinburgh Royal Infirmary during 1984 to 1991. They were stored as lyophilized cultures until required. All strains were identified by the API 20NE identification strip.

Antimicrobial agents. The following antibiotics were tested and obtained from the following companies: penicillin and ampicillin from Sigma Chemical Co. (Dorset, England), BRL 42715 and potassium clavulanate from SmithKline Beecham (Welwyn Garden City, England), piperacillin from Lederle Laboratories (Gosport, England), imipenem from Merck Sharp & Dohme (Hoddesdon, England), ceftazidime and cefuroxime from Glaxo Laboratories Ltd. (Uxbridge, England), cefotaxime and gentamicin from Roussel Laboratories Ltd. (Uxbridge, England), azlocillin and ciprofloxacin from Bayer (Newbury, England), and aztreonam from Bristol-Myers Squibb (Hounslow, England).

Antimicrobial susceptibility tests. MICs were determined by the agar dilution method. All assays were performed on Mueller-Hinton agar (Oxoid Ltd., Basingstoke, England). The inoculum used, approximately 10^4 CFU/ml, was applied with a Denley multipoint inoculator (Billinghurst, England). Plates were incubated overnight at 37°C. Each MIC was determined as the lowest concentration of antibiotic that inhibited visible growth.

IEF. Samples were applied to a polyacrylamide gel containing ampholines with a pH range of 3.5 to 10.6 or a 1:1 mixture of pH 3.5 to 10.6 and pH 4 to 6 as previously described (18). Isoelectric focusing (IEF) was carried out at 4°C at 1 W (constant), 500 V (limiting), and 20 mA (limiting) for 18 h. Gels were quantified with pI standard markers (BDH Ltd., Poole, England) and stained by overlaying the gels with filter paper soaked in nitrocefin (1 mM) either directly or after a 30-s overlay with various inhibitors (1 mM potassium clavulanate, 1 mM aztreonam, 1 mM cloxacillin, 1 mM EDTA, and 100 μM BRL 42715) soaked in filter paper as previously described (2).

Assessment of inducibility of β-lactamase. Ten-milliliter volumes of Mueller-Hinton broth were grown overnight at 37°C with continuous shaking. One milliliter of overnight culture was added to 9 ml of prewarmed Mueller-Hinton broth and incubated for a further 90 min. Either cefotaxime or

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TABLE 1. Susceptibilities of *X. maltophilia* isolates to various antibiotics

Strain	Yr isolated	MIC (μg/ml)[a]									
		AMP	PIP	AZL	CXM	CTX	CAZ	IMP	AZM	CIP	GEN
5B105	1984	>256	>256	>256	>256	128	>256	256	>256	4	64
6B52	1985	>256	128	16	>256	128	256	128	>256	8	128
6B133	1985	>256	>256	>256	>256	128	>256	>256	>256	8	128
6B295	1985	>256	>256	>256	>256	128	>256	256	>256	4	64
7B78	1986	>256	64	16	>256	64	128	256	256	4	4
12B286	1991	>256	128	32	>256	128	128	64	>256	4	256
12B346	1991	>256	256	256	>256	256	>256	128	>256	2	32

[a] Abbreviations: AMP, ampicillin; PIP, piperacillin; AZL, azlocillin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; IMP, imipenem; AZM, aztreonam; CIP, ciprofloxacin; GEN, gentamicin.

imipenem was then added at one-fourth the MIC. Control flasks with no added inducer were established. After incubation for a further 2 h, cells were harvested and washed and the β-lactamase was released by sonication; the procedure was repeated after 4 h. Enzyme activity was measured by UV spectrophotometry. Nitrocefin (100 μM) was used as the test substrate. Protein estimation was measured as previously described (34).

Large-scale preparation of β-lactamases. Mueller-Hinton broths (100 ml) were inoculated with the strain under study and grown overnight with shaking at 37°C. Cultures were then added to 900 ml of the same prewarmed broth, and incubation continued under the same conditions for 90 min. For induction, imipenem was then added at one-fourth the MIC. Incubation was continued for a further 3 h. After 3 h, bacterial cells were harvested by centrifugation at 4°C. The pellet was washed in 50 mM Tris HCl buffer (pH 8.0) and recentrifuged. The pellet was resuspended in 4 ml of the same buffer and disrupted by ultrasonication (8 μm for 1 min, three times) (MSE Soniprep 150; MSE Instruments, Crawley, United Kingdom) with constant cooling in an ice water bath. The preparation was partially purified by passing it through a Sephadex G-150 column (2 cm^2 by 90 cm) (Pharmacia, Uppsala, Sweden) previously equilibrated with 50 mM Tris HCl buffer (pH 8.0) containing 0.1 mM zinc sulfate. Samples were eluted with the same buffer with a flow rate of 12 ml/h. β-Lactamase activity was detected with the chromogenic cephalosporin nitrocefin. The molecular mass on this column was determined as previously described (1). The molecular weight standards were alcohol dehydrogenase (150,000), bovine serum albumin (66,000), and cytochrome *c* (12,384).

Purification of β-lactamase by electrodialysis. Five hundred microliters of the partially purified β-lactamase solution prepared from *X. maltophilia* 5B105 was applied to a preparative IEF gel containing pH 3.5 to 10.6 and pH 4 to 6 ampholines in a 1:1 ratio. After focusing as described above, a 1-cm-wide strip of filter paper soaked in nitrocefin solution was placed along either side of the gel from the anode to the cathode to determine the position of the focused β-lactamases. The β-lactamase bands were then excised from the gel and placed into a dialysis sack with a minimal amount of 50 mM sodium phosphate buffer (pH 7.0). The dialysis sack was placed in the cathode reservoir of a Mini Sub Cell (Bio-Rad Laboratories Ltd., Hemel Hempstead, England) previously filled with 50 mM sodium phosphate buffer (pH 7.0). A charge of 150 V was applied to the sack for 10 min. The dialysis sack was then removed, and the polyacrylamide gel discarded (28). The remaining purified β-lactamase preparation was reapplied to an IEF as described above.

SDS-free (native) PAGE. Sodium dodecyl sulfate (SDS)-free (native) polyacrylamide gel electrophoresis (PAGE) was performed on the Phastsystem (Pharmacia). The buffer system used on the strips was 0.88 M L-alanine–0.25 M Tris (pH 8.8). Buffer strips were made of 2% agarose IEF. Fractions eluted from the G-150 column that contained all β-lactamase bands (as determined by IEF) were pooled and concentrated to 5 ml in a centriprep 10 concentrator (Amicon, Danvers, Mass.). Four microliters of this solution was applied to a Phastgel homogenous 12.5 minigel. The separations were run as recommended by the manufacturer. β-Lactamase activity was visualized with nitrocefin as described above.

Determination of subunit size. The mass of the β-lactamase subunit was also estimated by SDS-PAGE (14). β-Lactamase extracts were treated with 5.0% β-mercaptoethanol and 2.5% SDS at 100°C for 5 min before electrophoresis on a Phastgel gradient 10 to 15 minigel according to the manufacturer's instructions. After electrophoresis, gels were incubated for 4 h in 50 mM Tris HCl buffer (pH 8.0) containing 1% Triton X-100 and 0.1 mM zinc sulfate to obtain a renaturation of enzymatic activity as described previously (17). After renaturation was complete, visualization of β-lactamase activity was obtained by staining with nitrocefin as described above. Low-molecular-weight protein standards (Bio-Rad Laboratories) were run simultaneously under the same conditions and stained with

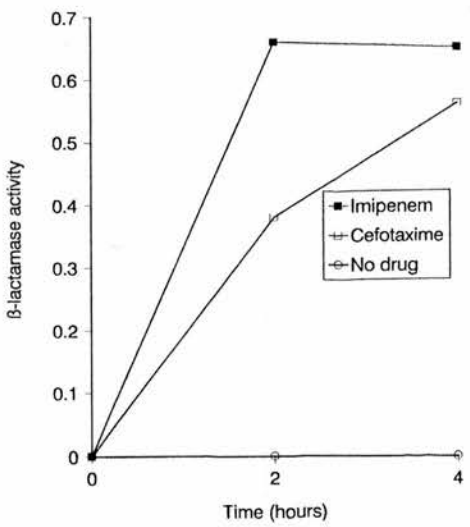

FIG. 1. Kinetics of β-lactamase induction for *X. maltophilia* 5B105. β-Lactamase activity is expressed as $\log_{10}$ nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.

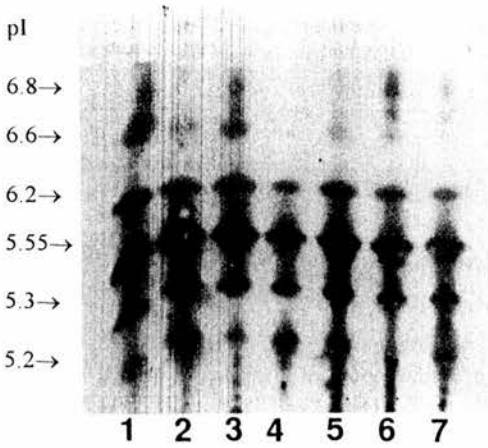

FIG. 2. IEF patterns of β-lactamases from seven strains of *X. maltophilia* isolated from blood cultures during 1984 to 1991. Lane 1, 5B105; lane 2, 6B52; lane 3, 6B133; lane 4, 6B295; lane 5, 7B78; lane 6, 12B286; lane 7, 12B346.

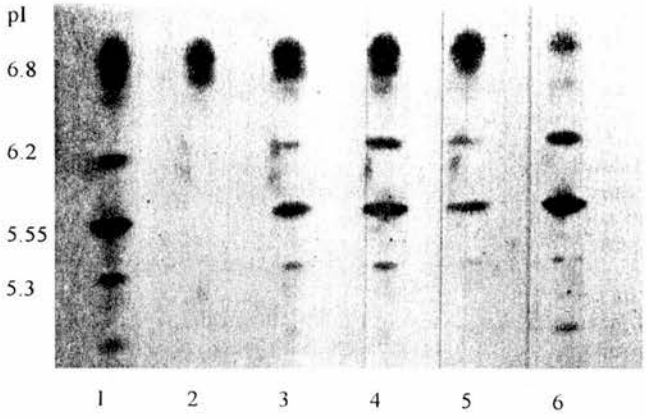

FIG. 3. IEF patterns of *X. maltophilia* β-lactamases overlaid with various inhibitors. Lane 1, control; lanes 2 to 6, overlaid with 100 μM BRL 42715, 1 mM aztreonam, 1 mM cloxacillin, 1 mM clavulanate, and 1 mM EDTA, respectively.

Coomassie brilliant blue R250. The molecular weight standards were transposed over the gel with the renatured β-lactamases for the molecular masses to be ascertained.

β-Lactamase assays. Assays of β-lactamase activity were performed at 37°C on a Perkin-Elmer λ 2 spectrophotometer with freshly prepared antibiotic solutions in either 50 mM Tris HCl buffer (pH 8.0) or 50 mM sodium phosphate buffer (pH 7.0) at the wavelength of maximal absorbance for the β-lactam ring of each drug over a 5-min period. The following wavelengths were used: 238 nm for penicillin and ampicillin, 236 nm for carbenicillin, 260 nm for cefuroxime and ceftazidime, 265 nm for cefotaxime, 240 nm for azlocillin, 255 nm for cephaloridine, and 299 nm for imipenem. Maximum rate of hydrolysis (V_{max}) and K_m values were derived by linear regression analysis of Lineweaver-Burk plots of initial velocity data at different substrate concentrations.

Inhibition studies. A solution of enzyme was preincubated with inhibitor for 5 min at 37°C in either 50 mM Tris HCl buffer (pH 8.0) or 50 mM sodium phosphate buffer (pH 7.0). The remaining enzymatic activity was assayed spectrophotometrically with cephaloridine (100 μM), imipenem (100 μM), or nitrocefin (100 μM) as the test substrate.

RESULTS

Antimicrobial susceptibilities. Table 1 shows the results of antimicrobial susceptibilities. All seven strains examined were resistant to almost all of the β-lactams tested, including imipenem. Only two strains (6B52 and 7B78) were susceptible to azlocillin (MIC, 16.0 μg/ml). All isolates were also resistant to gentamicin (breakpoint, 1.0 μg/ml [4]). Ciprofloxacin was the most effective agent of all compounds tested, with MICs for five of the seven strains tested either at or below the recommended breakpoint level (4 μg/ml) (4).

Inducibility of β-lactamase production. The kinetics of β-lactamase induction for strain 5B105 are shown in Fig. 1. The test substrate was nitrocefin. When imipenem or cefotaxime was added as an inducer, β-lactamase activity increased sharply after less than 1 h. Imipenem was a more potent inducer of β-lactamase than cefotaxime was. No discernible β-lactamase activity was detected in disrupted cells without the prior addition of an inducer. This result demonstrated that all β-lactamase activity was inducible. No enzyme activity was detected in the culture filtrate, indicating that the β-lactamase was intracellular.

IEF. β-Lactamase preparations from all seven strains exhibited identical IEF patterns and revealed the presence of at least four main bands of β-lactamase activity (pI 6.8, 6.2, 5.55, and 5.3) and two minor bands (pI 5.2 and 6.6) (Fig. 2). *X. maltophilia* 5B105 was selected as representative and examined in more detail.

Polyacrylamide gels were overlaid with various inhibitors to further elucidate the nature of these β-lactamases (Fig. 3). The β-lactamase band of pI 6.8 was inhibited by overlaying the gel with 1.0 mM EDTA prior to staining, whereas all other bands were unaffected. Conversely, the other β-lactamase bands were completely eliminated by the overlay of BRL 42715 and partially inhibited by potassium clavulanate. Aztreonam and cloxacillin overlays appeared to have no significant effect on any of the β-lactamase bands.

Gel filtration of crude β-lactamase. Spectrophotometric analysis of the fractions eluted from the Sephadex G-150 column and subsequent IEF (Fig. 4) revealed the presence of at least two distinct enzymes. The β-lactamase with a pI of 6.8 eluted first from the column and hydrolyzed imipenem as well as nitrocefin; the other β-lactamase bands eluted from the column at the same rate and hydrolyzed nitrocefin but not imipenem. The molecular masses of the enzymes were calcu-

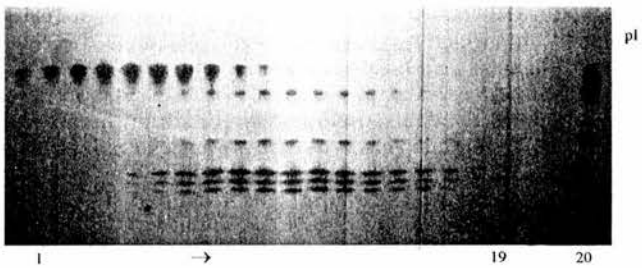

FIG. 4. IEF patterns of β-lactamases from *X. maltophilia* 5B105 eluted from a Sephadex G-150 column. Lanes 1 to 19, 15 μl of every second fraction eluted from a Sephadex G-150 column (fractions 52 to 88) applied to the gel; lane 20, concentrated β-lactamase preparation of *X. maltophilia* 5B105.

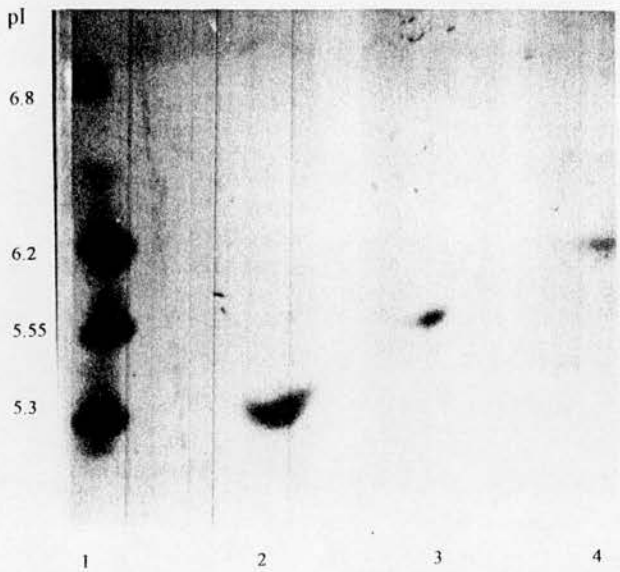

FIG. 5. IEF of β-lactamases from *X. maltophilia* 5B105 purified by electrodialysis. Lane 1, original β-lactamase preparation from 5B105; lanes 2 to 4, β-lactamase bands (pI 5.3, 5.55, and 6.2, respectively) purified by electrodialysis.

lated as 96 and 48 kDa, respectively, by comparing peak activity with the standard proteins.

In preliminary experiments, the β-lactamase of pI 6.8 was found to be unstable in 50 mM sodium phosphate buffer (pH 7.0) at 37°C and 80% of the activity was lost after 5 min of incubation in the buffer. The enzyme was stable in 50 mM Tris HCl with an optimum pH of 8.0. Therefore, all assays on the β-lactamase of pI 6.8 were performed with this buffer. The other β-lactamases were stable in phosphate buffer, and assays of these enzymes were performed with 50 mM sodium phosphate buffer (pH 7.0).

Purification of β-lactamase by electrodialysis. Purification of the three main β-lactamase bands (pI 5.3, 5.55, and 6.2) that were eliminated by BRL 42715 was performed by electrodialysis. Each band was excised from the gel, repurified, applied to an IEF gel, and reseparated (Fig. 5). Each preparation focused as a single band at exactly the same pI to which it had originally migrated, indicating that each β-lactamase was a discrete β-lactamase rather than a satellite band.

Native PAGE. Native PAGE of the concentrated β-lactamase preparation from the Sephadex G-150 gel column revealed the presence of four distinct bands of activity, compatible with the concept of four β-lactamases (Fig. 6).

SDS-PAGE. Two-milliliter fractions from the Sephadex G-150 column which contained either the β-lactamase of pI 6.8 or the β-lactamases which were inhibited by BRL 42715 were pooled and concentrated to 200 μl. SDS-PAGE analysis of the partially purified extracts followed by gel renaturation treatment (Fig. 7) showed in each case the presence of a single band of β-lactamase activity, with an apparent molecular mass of 26 kDa for the enzyme of pI 6.8, while the other β-lactamase bands showed the presence of a single protein with an apparent molecular mass of 24 kDa. Data suggest that the enzyme of pI 6.8 is a tetramer of four subunits which are very similar in molecular mass. Between them, the other β-lactamases exhibited a single band of β-lactamase activity by SDS-PAGE, indicating dimetric enzymes consisting of two subunits of similar size.

FIG. 6. Native PAGE of β-lactamases from *X. maltophilia* 5B105. Four microliters of the β-lactamase preparation was applied at the cathode. $^{+}$VE, anode; $^{-}$VE, cathode.

Hydrolysis of β-lactam antibiotics. Assays of β-lactamase hydrolysis of various β-lactams were performed with partially purified extracts from the G-150 column on the β-lactamase of pI 6.8. It was clearly evident at this stage that the β-lactamases of lower pI (<6.8) could not be separated, and they were subsequently pooled. For convenience, these β-lactamases are hereafter designated XM-A and XM-B, respectively (Table 2).

XM-A was primarily a penicillinase and also readily hydrolyzed imipenem. It was much less active against all classes of cephalosporins, although its affinities (low K_m values) for cephalosporins were higher than those for penicillins and imipenem. Aztreonam was not hydrolyzed. The XM-B enzymes exhibited a broad substrate profile, hydrolyzing an extensive range of β-lactams but not imipenem, ceftazidime, or aztreonam. Their affinities were higher for cephalosporins than for penicillins.

Inhibitors. Table 3 shows the effect of inhibitors on the activities of enzymes. The treatment of XM-A with EDTA resulted in the complete inhibition of enzymatic activity. This inhibition was completely reversed after dialysis against 50 mM Tris HCl (pH 8.0) for 30 min and the addition of 0.1 mM zinc sulfate to the reaction mixture. Enzymatic activity was partially inhibited by mercuric chloride and *p*-chloromercuribenzoate (PCMB) but not by either potassium clavulanate or BRL 42715 (50% inhibitory doses of >50 and >10 μM, respective-

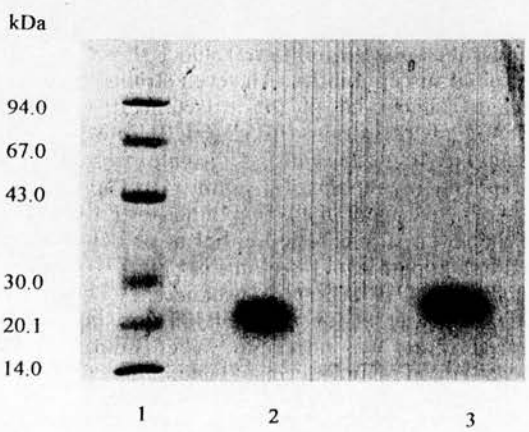

FIG. 7. SDS-PAGE of the β-lactamases from *X. maltophilia* 5B105. Lane 1, molecular mass standards; lane 2, β-lactamase bands of XM-B; lane 3, β-lactamase band of XM-A.

TABLE 2. Hydrolysis of β-lactam antibiotics by XM-A and XM-B β-lactamases from *X. maltophilia* 5B105

Substrate	XM-A			XM-B		
	V_{max}[a]	Relative V_{max}[b]	K_m (mM)	V_{max}[a]	Relative V_{max}[b]	K_m (mM)
Penicillin	14.3	100	0.6	1.2	100	0.108
Ampicillin	5.71	39.9	0.34	2.3	191.6	0.22
Carbenicillin	2.0	13.9	0.67	0.45	37.5	0.55
Azlocillin	5.5	38.4	0.24	1.25	104	0.22
Cephaloridine	0.09	0.6	0.22	1.8	150	0.05
Cefuroxime	0.66	4.6	0.133	0.28	23.3	0.076
Cefotaxime	0.303	2.1	0.05	0.19	15.8	0.08
Ceftazidime	0.03	0.2	0.09	NMH[c]		
Imipenem	3.33	23.3	0.25	NMH		
Aztreonam	NMH			NMH		

[a] Expressed as micromoles of substrate hydrolyzed per minute per milliliter of enzyme solution.
[b] Relative to the V_{max} of penicillin set at 100%.
[c] NMH, no measurable hydrolysis.

ly). The XM-B enzymes were not affected by the addition of 0.1 mM EDTA but were inhibited by both 1 mM potassium clavulanate and 100 μM BRL 42715 (50% inhibitory doses of 0.16 and <0.001, respectively). The enzymatic activity of XM-B was completely inhibited by the addition of 0.5 mM PCMB and 0.5 mM mercuric chloride (Table 3).

DISCUSSION

There have been few reports that have characterized the β-lactamases encoded by *X. maltophilia* in detail; it has been generally assumed that all isolates produce two β-lactamases, L1 and L2. Recent work, however, has revealed the heterogenicity of β-lactamase production by strains of *X. maltophilia*. These enzymes have not been characterized in full but have been differentiated only by virtue of their isoelectric points (9, 19).

All enzymatic activity observed was inducible. The production of two inducible β-lactamases is unusual, although it has previously been reported for strains of *Aeromonas* spp. (13), *Bacillus cereus* (10), and *Yersinia enterocolitica* (8). Some *Aeromonas* spp. have been reported to produce up to three different β-lactamases (32).

The multiple bands of β-lactamase activity observed with the strains examined in this report were difficult to purify to homogeneity. Repeated attempts to purify each band of β-lactamase activity were unsuccessful despite the utilization of ion-exchange chromatography with different pH buffers (i.e., above and below the pI of the β-lactamase bands). Not only did the distinct bands of β-lactamase fail to separate but the resulting enzymatic activity of the eluate was very poor. This technique was especially detrimental to the activity of the XM-A enzyme. However, the β-lactamase activity could be separated by gel filtration into two fractions, XM-A and XM-B.

TABLE 3. Effect of various inhibitors and ions on the activity of the XM-A and XM-B β-lactamases

Inhibitor	Concn (mM)	% Inhibition of:	
		XM-A[a]	XM-B[b]
PCMB	0.5	69	100
Mercuric chloride	0.5	70	100
EDTA	0.1	100	0

[a] Nitrocefin (100 μM) was used as the test substrate.
[b] Cephaloridine (100 μM) was used as the test substrate.

Polyacrylamide gel overlays with various inhibitors demonstrated that XM-A was inhibited by EDTA but not by BRL 42715 (a powerful inhibitor of serine-active-site β-lactamases [7]), indicating that this enzyme was distinct from the other bands of activity. Inhibitor overlays also demonstrated that the XM-B enzymes were inhibited by both BRL 42715 and potassium clavulanate.

The differences in the pIs of the XM-B bands suggest that they may have undergone amino acid substitution or addition away from the active site of the enzyme and may physically differ in their primary structure. Purification by electrodialysis and subsequent IEF demonstrated that each preparation focused as a single band of activity at exactly the same pI to which it had originally migrated. Its small yield of enzyme made this technique unsuitable for the purification of enzyme for further studies. Native PAGE of the concentrated partially purified β-lactamase preparation revealed the presence of four distinct bands of activity. Separated by gel filtration, all the bands that were inhibited by BRL 42715 were eluted at the same rate, with an apparent molecular mass of 48 kDa. When the molecular mass was estimated by SDS-PAGE and subsequent renaturation of β-lactamase activity, only one band with a molecular mass of 24 kDa was observed, suggesting a dimeric enzyme. It is improbable that an organism would encode three or more β-lactamases with identical molecular masses and subunit conformation unless they were closely related, like the TEM enzymes, and in parallel with the TEM β-lactamases, the differences in pIs arise from a few amino acid substitutions. These results indicated that the β-lactamase activity of XM-B was the result of at least three distinct enzymes and not of satellite bands. Unfortunately, the identical molecular masses of these enzymes and the similarities of their pIs made separation of these enzymes impossible, despite employing cation- and anion-exchange chromatography.

The XM-A enzyme was very similar in physical and biochemical profile to the L1 enzyme. It was unstable in sodium phosphate buffer. The plasmid-mediated metallo-β-lactamase from *Pseudomonas aeruginosa* is also unstable in phosphate buffer (35). The catalytic activity of the XM-A enzyme was dependent on Zn^{2+} for activity. Although the restoration of activity with other metal ions was not attempted in this study, results suggest that the enzyme is a metallo-β-lactamase. Both enzymes share similar pIs (L1, pI 6.9). In parallel with the L1 enzyme, the XM-A enzyme also exists as a tetramer in the active state (molecular mass, 96 kDa), with a subunit molecular mass of 26 kDa. The inhibitory profiles of both enzymes are very similar. Both are insusceptible to potassium clavulanate but susceptible to mercuric chloride. Iaconis and Sanders (13) reported that the L1-producing strain they examined was inhibited by PCMB (20%), whereas in 1982, Saino et al. (31) reported no inhibition by this compound. Under our test conditions, PCMB inhibited the enzymatic activity of the XM-A enzyme by 69%. The zinc-active sites of metallo-β-lactamases are thought to include a conserved cysteine residue (3); therefore, inhibition by PCMB would be expected. As with other metallo-β-lactamases (16), XM-A, although exhibiting predominantly penicillinase activity, also hydrolyzed a broad range of β-lactams including imipenem but not the monobactam aztreonam.

The other enzymes described in this report, XM-B, although differing particularly in their isoelectric points from that of the L2 enzyme described by Saino et al. (30) (pI 8.4), shared some similarities with this enzyme. However, it should be noted that

the results obtained with these combined enzymes should be treated minimally. We have shown that these enzymes have distinct physically properties (pIs); nevertheless, it has not been unequivocally shown that all these enzymes react in the same manner. The inhibitor profile of XM-B was similar to that reported for the L2 enzyme. All of these enzymes were inhibited by clavulanate, PCMB, and mercuric chloride. EDTA had no effect on the enzymatic activity of XM-B, indicating that XM-B does not require a divalent metal ion for catalytic activity. The inhibition by BRL 42715 suggests these enzymes are serine-active-site β-lactamases. The molecular mass of these enzymes was 48 kDa by gel filtration, whereas that of their subunit form was estimated to be 24 kDa by SDS-PAGE. The results show they are dimers in the native state, similar to the L2 enzyme (active-form molecular mass, 56 kDa; subunit molecular mass, 27 kDa). Together, the XM-B group of enzymes hydrolyzed a broad spectrum of antibiotics, including penicillins and cephalosporins, but not imipenem or aztreonam, whereas the L2 enzyme was primarily a cephalosporinase, with poor hydrolytic activity against penicillins.

Neither XM-A nor the XM-B group of enzymes was shown to hydrolyze aztreonam, although all the strains examined exhibited high levels of resistance to this drug. Another explanation of the high level of resistance to aztreonam may be the low permeability of the outer membrane of *X. maltophilia* to the influx of antibiotics (20).

Although the exact genetic locations of the genes which encode these β-lactamases have not been precisely determined, the inducibility of the enzymes would suggest that they are chromosomally mediated. Indeed, from this study and others (9, 27), there appears to be a paucity of plasmid-mediated β-lactamases in strains of *X. maltophilia*.

All β-lactamases are produced with a single inducer; this might imply that the enzymes share a common regulatory gene, although it is known that imipenem is an effective inducer for more than one β-lactamase (33).

We have shown that the XM-A enzyme shares biological and physiological properties with the L1 enzyme and clearly belongs in group 3 (metallo-β-lactamases [MET-N]) in the classification scheme of Bush (6); however, full sequencing is necessary for an absolute determination. The XM-B group of enzymes appears to be well placed in the Bush class 2b′ (extended-broad-spectrum β-lactamases inhibited by clavulanic acid [EBS-Y]) (5); however, without cloning the individual genes into a suitable host and subsequently sequencing the enzymes, it is not possible to determine whether these enzymes have diverse biochemical profiles. The XM-A and XM-B enzymes may be common among strains of *X. maltophilia*. Cullmann and Dick (9) reported the presence of six distinct β-lactamases in 19 strains of *X. maltophilia*. On close scrutiny of the IEF polyacrylamide gel, there appear to be β-lactamases from strain 858 that are similar to those described in this report, although Cullmann and Dick do not describe these enzymes in their text. Recently, Payne et al. (27) reported the presence of enzymes with similar pIs in two strains of *X. maltophilia*. It appears that within the species *X. maltophilia*, a number of different β-lactamases which differ from the β-lactamases of most other genera are produced.

Livermore (16) has classified carbapenemases as efficient or inefficient, depending on whether they confer high or low levels of carbapenem resistance on the host organism. High levels of carbapenem resistance have been found only in those organisms that are known to commonly produce a chromosomal metallo-β-lactamase. The metallo-β-lactamase of *X. maltophilia* is an example. There is also a frightening possibility that the chromosomal genes that encode the β-lactamases of this species may be transposed onto conjugative plasmids. There have been recent reports of inefficient carbapenemases, although not transferable, from *Acinetobacter baumannii* (26), *Enterobacter cloacae* (23), and *Serratia marcescens* (36), which are not of the normal β-lactamase complement for the host species. The increased use of β-lactam drugs, particularly the newer carbapenem compounds, may facilitate the emergence of this organism as an important pathogen. If the genes encoding these enzymes acquire the capability to disseminate among the more common pathogens, it will further diminish the range of antibiotics available for use in cases of serious sepsis.

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